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(54) METHOD FOR EVALUATING β CELL DYSFUNCTION IMPROVING AGENT

(57)Abstract:
PROBLEM TO BE SOLVED: To provide a new method for evaluating a β cell dysfunction improving agent, such as an insulin-resistant therapeutic agent and an insulin secretion promoting agent, by finding out a gene useful as an indicator of a symptom of diabetes and improvement thereof, especially, improvement of the β cell dysfunction, and utilizing the gene or a product of the gene, and to provide a new method for evaluating the β cell dysfunction of an examinee.
SOLUTION: The method for evaluating the β cell dysfunction improving effect of a test substance by using an expression level of the gene expressed by a specific base sequence or the product of the gene in a specimen under an administration condition of the test substance as the indicator, the method for evaluating the β cell dysfunction of the examinee, a primer for the methods, a kit containing a probe, or the like, are provided, respectively.

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CLAIMS

[Claim(s)]
[Claim 1]
How to evaluate the beta cell malfunction improvement effect of this examined substance by making into an index the gene shown according to any one base sequence of the array numbers 1-8 in the specimen under the administration conditions of an examined substance, or the amount of manifestations of the product (array numbers 9-16).
[Claim 2]
The approach according to claim 1 characterized by performing evaluation based on the difference with the gene shown according to any one base sequence of the array numbers 1-8 in the specimen under the administration conditions of an examined substance or the amount of manifestations of the product (array numbers 9-16), and the amount of manifestations concerned under the conditions of not prescribing a medicine for the patient.
[Claim 3]
How to evaluate the beta cell malfunction improvement effect including the following process of an examined substance :
1) Breed an animal under administration of an examined substance, or the conditions of not prescribing a medicine for the patient.;
2) Detect the amount of gene expression shown according to any one base sequence of the blood of the above-mentioned animal, or the array numbers 1-8 in a cell.;
3) Evaluate the beta cell malfunction improvement effect of this examined substance based on administration of an examined substance, and a difference of the above-mentioned amount of gene expression under the conditions of not prescribing a medicine for the patient.
[Claim 4]
The approach according to claim 3 of including further the process which extracts all RNA from the inside of blood or a cell in said approach.

[Claim 5]
The approach according to claim 3 or 4 characterized by detecting the amount of gene expression by any one approach chosen from a gene chip, a cDNA array and the nucleic acid hybridization method using the solid phase-ized sample chosen from a membrane filter, RT-PCR method, the real-time PCR method, a subtraction technique, the differential displaying method, a differential hybridization method, and a cross hybridization method.

[Claim 6]
The approach according to claim 3 or 4 that the amount of gene expression is characterized by being detected by RT-PCR method or the real-time PCR method.
[Claim 7]
How to evaluate the beta cell malfunction improvement effect including the following process of an examined substance :
1) Breed an animal under administration of an examined substance, or the conditions of not prescribing a medicine for the patient.;
2) Detect the protein shown according to any one amino acid sequence of the blood of the above-mentioned animal, or the array numbers 9-16 in a cell using the antibody specifically combined with this protein.;
3) Evaluate the beta cell malfunction improvement effect of this examined substance based on a difference of the amount of manifestations of administration of an examined substance and the above-mentioned protein under the conditions of not prescribing a medicine for the patient.
[Claim 8]
The approach according to claim 7 characterized by detecting the amount of manifestations of protein by any one approach chosen from a Western blot technique, dot blotting methods, slot blotting methods, the ELISA method, and the RIA method.
[Claim 9]
The approach according to claim 7 that the amount of manifestations of protein is characterized by being detected by the Western blot technique.
[Claim 10]
An approach given in any 1 term of claims 3-9 whose cell is a pancreas Langerhans Mr. island beta cell.
[Claim 11]
An approach given in any 1 term of claims 3-10 whose animal is a type 2 diabetes model animal.
[Claim 12]
The approach according to claim 11 an animal is a mouse.
[Claim 13]
How to evaluate the beta cell malfunction improvement effect including the

following process of an examined substance :
1) Cultivate a cell under addition of a specimen material, or the conditions of not adding.;
2) Detect the amount of gene expression shown according to any one base sequence of the array numbers 1-8 in the above-mentioned cell, or detect the amount of manifestations of the protein (array numbers 9-16) which is the product using the antibody specifically combined with this protein.;
3) Evaluate the beta cell malfunction improvement effect of this examined substance based on a difference of addition of a specimen material and the above-mentioned gene under the conditions of not adding, or the amount of manifestations of protein.
[Claim 14]
An approach given in any 1 term of claims 1-13 whose beta cell malfunction improvement effect is a thing based on an insulin resistance improvement effect.
[Claim 15]
How to evaluate this test subject's beta cell malfunction by measuring the amount of manifestations of the HITOOSOROGU product of the gene shown according to any one base sequence of the array numbers 1-8 in the blood isolated from the test subject.
[Claim 16]
The approach according to claim 15 characterized by detecting the amount of manifestations of protein by any one approach chosen from a Western blot technique, dot blotting methods, slot blotting methods, the ELISA method, and the RIA method.
[Claim 17]
The approach according to claim 15 that the amount of manifestations of protein is characterized by being detected by the Western blot technique.
[Claim 18]
The beta cell malfunction improvement effect containing at least one or more chosen from the group which consists of following a-e, or the kit for evaluation of a beta cell malfunction.
a) The oligonucleotide primer which 15 - 30 base length for amplifying specifically the gene shown according to any one base sequence of the array numbers 1-8 followed
b) The polynucleotide probe with which the 20 - 1500 base length for combining with the gene shown according to any one base sequence of the array numbers 1-8 specifically, and detecting this gene continued
c) The solid phase-ized sample to which the polynucleotide probe given [above-mentioned] in b was fixed
d) The antibody for combining with the protein shown according to any one amino acid sequence of the array numbers 9-16 specifically, and detecting

this protein
e) The second antibody which can be specifically combined with an antibody given [above-mentioned] in d

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[Translation done.]

DETAILED DESCRIPTION

[Detailed Description of the Invention]
[Field of the Invention]
[0001]
This invention relates to the kit for the approach for evaluation of the beta cell malfunction improvement agent using the gene used as the index of the beta cell malfunction improvement in diabetes mellitus, or its product, or a beta cell malfunction, and this approach.
[Background of the Invention]
[0002]
In recent years, in Japan, the diabetic is increasing rapidly, it is presumed that the number amounts to 7 million people, and diabetes mellitus serves as a disease with frequency high subsequently at hypertension. Especially 95% of a Japanese diabetic has type 2 diabetes (insulin non-dependency diabetes mellitus, NIDDM), and early detection and early treatment are important for it from the point of the prognosis.
[0003]
The symptoms of type 2 diabetes are shown according to the various origins, such as a hereditary predisposition and an environmental factor. As a cause in type 2 diabetes that an insulin operation is insufficient, the abnormalities (insulin resistance) of an insulin susceptibility device and the fall of insulin secretion are cited. In Europe and America, although the former, i.e., insulin resistance, is the main causes of type 2 diabetes, there are not little many in Japan, also when insulin hyposecretion is the main causes.
[0004]
The pancreas consist of an external secretion system cell (external secretion enzymes, such as an amylase and lipase, are produced) and an endocrine system cell (an insulin, glucagon, etc. are produced), and the latter forms the organization called the Langerhans Mr. island which consists of an alpha cell (glucagon is produced), a beta cell (an insulin is produced), etc. Among these, a beta cell occupies about 90% of a Langerhans Mr. islet cell, and since it has the work to which the blood sugar level is reduced by compounding and secreting an insulin, the malfunction brings about the fall of insulin production, and the fall of insulin secretion, and makes the symptoms of diabetes mellitus show.
[0005]
In type 1 diabetes, in order that a beta cell may be completely destroyed by the immune system and an insulin production cell may run short, the insulin replacement therapy is used for a therapy. On the other hand, in type 2 diabetes, when a target tissue shows insulin resistance to glucose incorporation, the function of a beta cell falls and a beta cell malfunction is produced soon.
[0006]
By the way, the glucose in blood is stimulated and produces secretion of the insulin in a beta cell. On the film of a beta cell, a glucose is incorporated by intracellular through the glucose transporter (GLUT2) which carries out localization, and is introduced by glucokinase to glycolytic pathway. And ATP produced in the metabolic turnover process of this glucose functions as an insulin secretion signal in a beta cell.

[0007]
The increment in concentration intracellular [ATP] produced by glucose metabolism closes the ATP susceptibility potassium channel (KATP) discovered by Cook and others in 1984, and it causes the depolarization of a cell membrane (for example, nonpatent literature 1 reference). It continues at this, a potential dependency calcium channel carries out opening, and the inflow of the calcium ion into a beta cell takes place. The rise of intracellular calcium ion concentration activates the insulin secretion exocytosis reaction of a calcium dependent (for example, nonpatent literature 2 reference).
[0008]
As a remedy of the type 2 diabetes which promotes insulin secretion, the sulfonyl urea agent is used widely conventionally. Although these drugs promote insulin secretion by closing the KATP channel of a beta cell, they have the fault of promoting insulin production and secretion regardless of the blood sugar level. Therefore, a food intake must be controlled by this kind of drugs, being cautious of hypoglycemia.
[0009]
On the other hand, it is Glucagon-like peptide -1 (GLP-1) as a type 2 diabetes medicine new now. It is developed. GLP-1 -- intestinal tract preproglucagon from -- although it is the amino acid produced by posttranslational modification -- a sulfonyl urea agent -- differing -- the blood sugar level -- promoting insulin secretion anaclitic is reported (for example, nonpatent literature 3 reference). GLP-1 not only promotes insulin secretion, but it promotes normalization of the blood sugar level by checking emission of glucagon. Furthermore, GLP-1 promotes the gene expression of hexokinase and increasing the biosynthesis of an insulin is also reported (for example, nonpatent literature 4 reference).
[0010]
Now, as drugs which promote insulin secretion, there are no effective drugs in addition to sulfonyl urea [which was mentioned above] and GLP-1. Therefore, development of the new remedy to a type 2 diabetes patient's beta cell malfunction is desired.
[0011]
On the other hand, the insulin resistance improvement agent has been developed as a remedy of the type 2 diabetes which normalizes an insulin susceptibility device. "Insulin resistance improvement agents" is drugs which raise the susceptibility of an insulin operation, compensate the lack of an operation of an insulin, and improve insulin resistance by reinforcing the activity of the tyrosine kinase which manages signal transfer of an insulin receptor. As such an insulin resistance improvement agent For example, troglitazone (for example, patent reference 1 reference), pioglitazone (For example, the patent reference 2 - 4 reference), ROSHIGURITAZON (for example, five to patent reference 7 reference), GI-262570 (for example, patent reference 8 reference), JTT-501 (Patent reference 9 - 11 reference [for example,]) AZ-242 (for example, 12 to patent reference 14 reference), MCC-555 (for example, 15 to patent reference 17 reference), YM-440 (Patent reference 18 - 20 reference [for example,]) KRP-297 (for example, the patent reference 21 and 22 reference), T-174 (for example, 23 to patent reference 25 reference), NC-2100 (Patent reference 26 - 28 reference [for example,]) NN-622 (for example, the patent reference 29 and 30 reference), An oxazole compound like BMS-298585 (for example, patent reference 31 reference), an OKISA diazo lysine compound, a thiazolidine compound, or a phenoxazine compound can be mentioned.
[0012]
Thiazolidine derivative like troglitazone, ROJIGURITAZON, and pioglitazone among the above-mentioned insulin resistance improvement agents is already used at the clinical place (for example, nonpatent literature 5-7 and patent reference 32 reference). These are considered to improve a type 2 diabetes patient's insulin resistance by being characterized by having a thiazolidine ring frame as basic structure, using as a target molecule PPARgamma which is a receptor in a nucleus, and changing the gene expression in liver, muscles, and a fat cell.
[0013]
In recent years, quick and comprehensive gene expression analysis is attained by advance of

molecular biology using the differential displaying method or the microarray method. The gene which serves as the target needs to be identified for development of the new remedy to a type 2 diabetes patient's beta cell malfunction. However, about beta cells of pancreas, although gene expression analysis in connection with the property and function of a beta cell is performed using the culture cell strain, comprehensive analysis of the gene relevant to the diabetic onset or a diabetic improvement is not performed.

[0014]

[Patent reference 1] JP,60-051189,A
[Patent reference 2] JP,61-267580,A
[Patent reference 3] The Europe patent No. 193,256 specification
[Patent reference 4] U.S. Pat. No. 4,687,777 specification
[Patent reference 5] Patent Publication Heisei No. 512249 [nine to] official report
[Patent reference 6] International public presentation/[95th] No. 21608 pamphlet
[Patent reference 7] U.S. Pat. No. 5,002,953 specification
[Patent reference 8] International public presentation/[00th] No. 8002 pamphlet
[Patent reference 9] International public presentation/[95th] No. 18125 pamphlet
[Patent reference 10] The Europe public presentation No. 684,242 specification
[Patent reference 11] U.S. Pat. No. 5,728,720 specification
[Patent reference 12] International public presentation/[99th] No. 62872 specification
[Patent reference 13] The Europe patent application public presentation No. 1,084,103 specification
[Patent reference 14] U.S. Pat. No. 6,258,850 specification
[Patent reference 15] JP,6-247945,A
[Patent reference 16] The Europe patent application public presentation No. 604,983 specification
[Patent reference 17] U.S. Pat. No. 5,594,016 specification
[Patent reference 18] International public presentation/[94th] No. 25448 pamphlet
[Patent reference 19] The Europe patent No. 696,585 specification
[Patent reference 20] U.S. Pat. No. 5,643,931 specification
[Patent reference 21] JP,10-87641,A
[Patent reference 22] U.S. Pat. No. 5,948,803 number specification
[Patent reference 23] JP,64-56675,A
[Patent reference 24] The Europe patent No. 283035 specification
[Patent reference 25] U.S. Pat. No. 4,897,393 specification
[Patent reference 26] JP,9-100280,A
[Patent reference 27] The Europe patent application public presentation No. 787725 specification
[Patent reference 28] U.S. Pat. No. 5,693,651 specification
[Patent reference 29] International public presentation/[99th] No. 19313 pamphlet
[Patent reference 30] U.S. Pat. No. 6,054,453 number specification
[Patent reference 31] International public presentation/[01st] No. 21602 pamphlet
[Patent reference 32] JP,60-051189,A
[Nonpatent literature 1] "Nature (Nature)" (1984) 311 p271-273
[Nonpatent literature 2] "Physio logy review (Physiological Revue)" (1981) 61 p914-973
[Nonpatent literature 3] "Die ABETISU (Diabetes)" (1994) Apr., 43(4):535-9
[Nonpatent literature 4] "Endocrinology (Endocrinology) Nov;136(11), p4910-7" (1995)
[Nonpatent literature 5] "Life science (Life Science) 67 p2405-2416" (2000)
[Nonpatent literature 6] "A Japanese clinical" (2000) 58 p389-404
[Nonpatent literature 7] "Pharma KOSERAPI (Pharmacotherapy) 21 p1082-1099" (2001)

[Description of the Invention]

[Problem(s) to be Solved by the Invention]

[0015]

This invention specifies the gene which participates in diabetic symptoms, its improvement, especially an improvement of a beta cell malfunction, and aims at offering the kit for the evaluation system of the diabetic medicine or diabetes mellitus using this, and this evaluation system.

[Means for Solving the Problem]
[0016]
In order that this invention persons may solve the above-mentioned technical problem, as a result of inquiring wholeheartedly, I thought that the gene with which the manifestation is increasing by a diabetic's pancreas Langerhans Mr. islet cell, and the amount of manifestations is normalized by administration of diabetic medicine could become diabetic symptoms and the index of the improvement. And by analyzing the amount of manifestations of these genes or its product, a header and this invention were completed for diabetic symptoms and evaluation of the improvement being possible.
[0017]
Namely, this invention offers the approach of evaluating the beta cell malfunction improvement effect of this examined substance, by making into an index the gene shown according to any one base sequence of the array numbers 1-8 in the specimen under the administration conditions of an examined substance, or the amount of manifestations of the product (array numbers 9-16).
[0018]
It may set to the above-mentioned approach and evaluation may carry out comparative evaluation of the difference of said gene under administration of an examined substance and the conditions of not prescribing a medicine for the patient, or the amount of manifestations of the product.
[0019]
In one embodiment [I], the approach of this invention includes the following process.
1) Breed an animal under administration of an examined substance, or the conditions of not prescribing a medicine for the patient.;
2) Detect the amount of gene expression shown according to any one base sequence of the blood of the above-mentioned animal, or the array numbers 1-8 in a cell.;
3) Evaluate the beta cell malfunction improvement effect of this examined substance based on administration of an examined substance, and a difference of the above-mentioned amount of gene expression under the conditions of not prescribing a medicine for the patient.
[0020]
Here, said process 2 may detect the amount of gene expression from all these RNA further including the process which extracts all RNA from the inside of blood or a cell.
[0021]
Said amount of gene expression can be detected by any one approach chosen from a gene chip, a cDNA array and the nucleic acid hybridization method using the solid phase-ized sample chosen from a membrane filter, RT-PCR method, the real-time PCR method, a subtraction technique, the differential displaying method, a differential hybridization method, and a cross hybridization method, and RT-PCR method and its real-time PCR method are especially desirable.
[0022]
Moreover, in another embodiment [II], the approach of this invention includes the following process.

1) Breed an animal under administration of an examined substance, or the conditions of not prescribing a medicine for the patient.;
2) Detect the protein shown according to any one amino acid sequence of the blood of the above-mentioned animal, or the array numbers 9-16 in a cell using the antibody specifically combined with this protein.;
3) Evaluate the beta cell malfunction improvement effect of this examined substance based on a difference of the amount of manifestations of administration of an examined substance and the above-mentioned protein under the conditions of not prescribing a medicine for the patient.
[0023]
Here, the amount of manifestations of said protein can be detected by any one approach chosen from a Western blot technique, dot blotting methods, slot blotting methods, the ELISA method, and the RIA method, and especially its Western blot technique is desirable.
[0024]
As for a cell, in said mode [I] and mode [II], it is desirable to use a pancreas Langerhans Mr. island

beta cell.
Moreover, as for an animal, it is desirable to use a type 2 diabetes model animal, especially a type 2 diabetes model mouse.
[0025]
Furthermore, in another embodiment [III], the approach of this invention includes the following process.
1) Cultivate a cell under addition of a specimen material, or the conditions of not adding.;
2) Detect the amount of gene expression shown according to any one base sequence of the array numbers 1-8 in the above-mentioned cell, or detect the amount of manifestations of the protein (array numbers 9-16) which is the product using the antibody specifically combined with this protein.;
3) Evaluate the beta cell malfunction improvement effect of this examined substance based on a difference of addition of a specimen material and the above-mentioned gene under the conditions of not adding, or the amount of manifestations of protein.
[0026]
In said mode [III], the cell of a cell which said gene and its product have high-discovered reflecting the beta cell malfunction symptoms in diabetes mellitus is desirable.
[0027]
This invention offers the approach of evaluating this test subject's beta cell malfunction, by measuring the amount of manifestations of the HITOOSOROGU product of the gene shown again according to any one base sequence of the array numbers 1-8 in the blood isolated from the test subject.
[0028]
Here, the amount of manifestations of said protein can be detected by any one approach chosen from a Western blot technique, dot blotting methods, slot blotting methods, the ELISA method, and the RIA method, and especially its Western blot technique is desirable.
[0029]
This invention offers the kit for evaluation of the beta cell malfunction improvement effect an examined substance's, or a test subject's beta cell malfunction again. This kit contains at least one or more chosen from the group which consists of following a-e.
a) The oligonucleotide primer which 15 - 30 base length for amplifying specifically the gene shown according to any one base sequence of the array numbers 1-8 followed
b) The polynucleotide probe with which the 20 - 1500 base length for combining with the gene shown according to any one base sequence of the array numbers 1-8 specifically, and detecting this gene continued
c) The solid phase-ized sample to which the polynucleotide probe given [above-mentioned] in b was fixed
d) The antibody for combining with the protein shown according to any one amino acid sequence of the array numbers 9-16 specifically, and detecting this protein
e) The second antibody which can be specifically combined with an antibody given [above-mentioned] in d
In addition, in the method of evaluating the above-mentioned beta cell malfunction improvement effect, and the kit for it, this beta cell malfunction improvement effect may be effectiveness brought about by improvement of insulin resistance.
[Effect of the Invention]
[0030]
It was shown by this invention that the amount of gene expression shown according to any one base sequence of the array numbers 1-8 can serve as a new index for evaluating the beta cell malfunction in diabetes mellitus and its improvement. Therefore, simple screening of beta cell malfunction improvement agents, such as an index, then an insulin resistance improvement agent, an insulin secretion accelerator, or a diagnosis of a beta cell malfunction can be performed for the amount of manifestations of this gene or its product.
[Best Mode of Carrying Out the Invention]

[0031]
Hereafter, this invention is explained to a detail.
This invention relates to the approach of evaluating the beta cell malfunction improvement effect of an examined substance, or a test subject's beta cell malfunction, by making into an index the gene shown according to any one base sequence of the array numbers 1-8, or the amount of manifestations of the product (array numbers 9-16).
[0032]
1. Target Gene
The gene (henceforth a "target gene") used by the approach of this invention is a gene which can serve as an index of the beta cell malfunction improvement in diabetes mellitus. Such a gene can be identified as a gene by which the amount of gene expression concerned is normalized in connection with the manifestation increasing remarkably on a diabetic's pancreas Langerhans Mr. island, and a beta cell malfunction improving.
[0033]
For example, said gene can be identified according to the following processes.
1) First prepare all RNA of the pancreas Langerhans Mr. island origin of a diabetic, healthy people or a diabetes-mellitus model animal, and the normal animal.;
2) subsequently, above-mentioned every -- a total --; which extracts the gene from which cRNA or cDNA is prepared and the amount of manifestations differs intentionally from RNA between a diabetic and healthy people or between a diabetes-mellitus model animal and the normal animal
3) Choose the gene by which the amount of manifestations is normalized as a target gene with the improvement of beta cell malfunction symptoms by prescribing beta cell malfunction improvement agents (for example, an insulin resistance improvement agent, an insulin secretion accelerator, etc.) for the patient among the extracted genes.
[0034]
The adjustment approach of the above-mentioned in process one, all RNA, cRNA, or cDNA and the analysis approach of the amount of gene expression are explained to a detail in the following term "2.1 The evaluation approach (inch vivo system) of the examined substance which made the target gene the index."
[0035]
In this way, this invention persons identified the following eight genes shown according to any one base sequence of the array numbers 1-8 as a target gene of this invention. In addition, the gene relation with diabetes mellitus is already suggested, saying was excepted in selection of a gene. Therefore, the target gene of this invention is completely strange about relevance with diabetes mellitus, although the array is already well-known.
[0036]
The target gene shown according to any one base sequence of the array numbers 1-8 carries out the code of the protein shown according to any one amino acid sequence of the array numbers 9-16, respectively (refer to [of an example 3 / Table 2]). Other information about each gene and its protein can come to hand from gene databases, such as GenBank and DDBJ.
[0037]
Although the above-mentioned target gene is specified for convenience in this invention according to the base sequence shown in the array numbers 1-8, this gene is not limited to these base sequences. It is contained in the target gene of this invention, as long as it has an array similar to the gene the gene is indicated to be to the array numbers 1-8 and has an equivalent function. Therefore, the OSOROGU (ortholog) is contained in the target gene shown according to any one base sequence of the array numbers 1-8. "OSOROGU" is an OSOROGASU gene (orthologous gene) and means evolutionally the gene of a different kind with which structure and a function were similar with the same origin here. Similarly, the protein in which a code is carried out by said OSOROGU besides the protein shown by the array numbers 9-16 shall also be included in the protein used by this invention.
[0038]
As shown below, HITOOSOROGU of the gene shown by the array numbers 1-8 carries out the

code of the protein which has the base sequence shown by the array numbers 49-56, and has the amino acid sequence shown by the array numbers 57-64. These HITOOSOROGU and an OSOROGU product are used for a diagnosis of the beta cell malfunction in Homo sapiens.
[0039]
Mouse HITOOSOROGU
GenBank Gene Protein GenBank Gene Protein
AK005296 Array number 1 Array number 9 BC012531 Array number 49 Array number 57
AK005484 Array number 2 Array number 10 NM_030968.1 Array number 50 Array number 58
NM_021434 Array number 3 Array number 11 NM_180989 Array number 51 Array number 59
AK006207 Array number 4 Array number 12 AF151877 Array number 52 Array number 60
BC010831 Array number 5 Array number 13 NM_018487 Array number 53 Array number 61
AK013996 Array number 6 Array number 14 NM_024006 Array number 54 Array number 62
AF272044 Array number 7 Array number 15 NM_030926 Array number 55 Array number 63
NM_007786 Array number 8 Array number 16 NM_005212 Array number 56 Array number 64
[0040]
2. Evaluation Approach of Beta Cell Malfunction Improvement Effect
The approach of this invention is an approach of evaluating the beta cell malfunction of this examined substance by making into an index the gene shown according to any one base sequence of the array numbers 1-8 in the specimen under the administration conditions of an examined substance, or the amount of manifestations of the product (array numbers 9-16).
[0041]
Said approach may measure and evaluate the administration and the target gene in the specimen under the conditions of not prescribing a medicine for the patient, or the amount of manifestations of the product, about one examined substance, and may perform comparative evaluation same about two or more examined substances. Or as long as a target gene or the amount of manifestations of the product, and correlation of a beta cell malfunction improvement effect are established experientially, based on the relation, the beta cell malfunction improvement effect of an examined substance may be evaluated without comparison contrast on an absolute scale.
[0042]
Moreover, the approach of this invention may carry out independent evaluation of one gene chosen from eight target genes, or the amount of manifestations of the product, and may evaluate two or more genes chosen from eight target genes, or the amount of manifestations of those products (manifestation profile) on the whole.
[0043]
In the evaluation approach of this invention, a beta cell malfunction improvement effect may evaluate the amount of target gene expression as an index, and may evaluate the amount of manifestations of the product of the gene concerned as an index. Furthermore, an evaluation system may be an in vivo system which used the animal, and may be an in vitro system using a cultured cell.
[0044]
In addition, in this invention, a "beta cell malfunction" means that a pancreas Langerhans Mr. island beta cell loses the normal function. The various diabetes-mellitus symptoms which specifically include the rise of the blood sugar level brought about by the abnormalities (the so-called insulin resistance) of an insulin susceptibility device, the fall of insulin secretion, and these are meant.
[0045]
In this invention, a "specimen" means the sample in which the target gene of this invention is contained for the blood isolated from a cultured cell, its extract, or an animal, body fluid, an organization, a cell, excrement, or those extracts. In especially this invention, the cell which blood, or a target gene and its product have high-discovered is desirable, therefore a pancreas Langerhans Mr. islet cell, especially its beta cell are the most desirable.
[0046]
Moreover, in this invention, "administration" for a living thing, "addition" to culture medium, etc.

shall contain all of making the condition that an examined substance exists in a specimen with "administration" of an examined substance.
Furthermore, the vocabulary a "gene" shall contain both 2 chains and a single strand not only including DNA but including RNA, cDNA(s), and all the cRNA(s).
[0047]
2.1 Evaluation Approach of Examined Substance Which Made Target Gene Index (Inch Vivo System)
As for the evaluation approach of the beta cell malfunction improvement effect of the examined substance in in vivo which made the target gene the index, it is desirable to include the following process.
[0048]
Process 1: Breed an animal on condition that administration of an examined substance, or un-prescribing a medicine for the patient.
Process 2: Detect the amount of target gene expression in the blood of the above-mentioned animal, or a cell.
Process 3: Evaluate the beta cell malfunction improvement effect of this examined substance based on administration of an examined substance, and a difference of the amount of target gene expression under the conditions of not prescribing a medicine for the patient.
[0049]
Process 1: Breeding of an animal
Although especially the "animal" used by the approach of this invention is not limited, the type 2 diabetes model animal which presents a beta cell malfunction is desirable. Even if such an animal is a commercial thing, it may be produced according to a well-known approach. As a commercial type 2 diabetes model animal, type 2 diabetes model rats, such as type 2 diabetes model mice, such as KK mice (for example, a KK/Ta mouse, a KK/San mouse, etc.), KK-Ay mice (for example, KK-Ay/Ta mouse etc.), a C57 BL/KsJ db/db mouse, C57BL / 6 J db/db mouse, and an ob/ob mouse, and GK rat, etc. can be mentioned, for example. These mice and rats can be purchased for example, from Japanese Clare, Inc.
[0050]
Said animal performs administration of an examined substance, or period breeding suitable under the conditions of not prescribing a medicine for the patient. Especially the dose of the examined substance to an animal is not limited, but is suitably set up according to the description of an examined substance, or the weight of an animal. Moreover, what is necessary is not to limit especially the medication method and administration period of an examined substance to an animal, either, but just to set up suitably according to the description of an examined substance.
[0051]
Process 2: Detection of a target gene
Next, blood or a cell is isolated from the animal bred under administration of an examined substance, or the conditions of not prescribing a medicine for the patient, and the amount of target gene expression in this blood or a cell is detected.
As a cell made applicable to detection, the cell which a target gene and its product have high-discovered is desirable, therefore a pancreas Langerhans Mr. islet cell, especially its beta cell are desirable.
[0052]
As the detection approach of a target gene, all RNA can be first extracted from the isolated blood or the cell, for example, and the approach of detecting the amount of manifestations of the target gene (mRNA) in [this all] RNA can be mentioned.
[0053]
(1) The extract of all RNA
The extract of all RNA is extracted using the solvent for an RNA extract according to a well-known approach from the isolated blood or the cell. The things (for example, a TRIzol reagent: product made from Gibco BIARUERU etc.) containing the component which has the operation which inactivates RNase, such as a phenol, as this extracting solvent, for example are desirable.

Especially the extract approach of RNA is not limited, for example, can adopt thiocyanic acid guanidine and a cesium chloride ultracentrifugal method, a thiocyanic acid guanidine hot phenol process, a guanidine hydrochloric-acid method, the acid thiocyanic acid guanidine phenol chloroform method (Chomczynski, P.and Sacchi, N. (1987), Anal.Biochem., 162, 156-159), etc. Especially, the acid thiocyanic acid guanidine phenol chloroform method is suitable.

[0054]

All extracted RNA may be further refined and used only for mRNA if needed. Although especially the purification approach is not limited, since it has the Pori (A) array in that three-dash terminal, many of mRNA(s) which exist in the cytoplasm of an eukaryotic cell can be carried out as follows, using this description. First, a biotin-ized oligo (dT) probe is added to all extracted RNA, and (Pori A) +RNA is made to adsorb. Next, the paramagnetism particle support which fixed streptoavidin is added and (Pori A) +RNA is made to catch using association between a biotin/streptoavidin. (Pori A) +RNA is eluted from an oligo (dT) probe at the last after washing actuation. (Pori A) +RNA may be made to adsorb using an oligo (dT) cellulose column besides this approach, and the approach of it being eluted and refining this may also be adopted. Fractionation of the (Pori A) +RNA by which elution was carried out may be further carried out with a sucrose density-gradient centrifugation method etc.

[0055]

(2) Detection of a target gene

Next, the amount of target gene expression in [all / under administration of an examined substance or the conditions of not prescribing a medicine for the patient] RNA is detected. The amount of gene expression is detectable as the signal reinforcement by preparing cRNA or cDNA and carrying out the label of this with a suitable labeled compound from all obtained RNA.

[0056]

Hereafter, it divides into the analysis approach and the iiRT-PCR method (real-time PCR method) which used i solid phase-ized sample, and the analysis approach of iii and others, and the detection approach of the amount of gene expression is explained concretely.

[0057]

i) The analysis approach using a solid phase-ized sample

It mixes in the solid phase-ized sample which fixed the well-known gene separately on the same conditions, and it is made to hybridize to coincidence cDNA or cRNA (henceforth an "indicator probe") under administration of an examined substance, or the conditions of not prescribing a medicine for the patient which carried out the indicator (Brown, P.O.et al.(1999) Nature genet.21, suppliment, 33-37). That to which the mRNA clone of a target gene also carried out the indicator of all the discovered mRNA(s) is sufficient as said indicator probe. Although mRNA which is not refined may be used as a start ingredient for probe production, it is more desirable to use (Pori A) +RNA refined by the above-mentioned approach. As a solid phase-ized sample, the following can be mentioned, for example.

[0058]

a) Gene chip :

As long as the target gene which is a candidate for detection is solid-phase-ized, the gene chip used by this invention may be produced based on a well-known approach (Lipshutz, R.J.et al.(1999) Nature genet.21, suppliment, 20-24), even if it is a commercial thing. For example, as a commercial gene chip with which the gene of a mouse was fixed, mouse MG-U74 (U74A, U74B, U74C, U74v2) by the AFI metrics company etc. can be mentioned.

[0059]

The detection and analysis by the gene chip can be carried out according to a conventional method. For example, if it is the case where the chip by the AFI metrics company is used, according to the protocol attached to the product, the cRNA probe which carried out the biotin indicator will be prepared. Subsequently, hybridization is performed according to this protocol, and if luminescence by avidin is detected and analyzed, the amount of gene expression can be calculated.

[0060]

b) An array or membrane filter :
As long as the target gene which is a candidate for detection is solid-phase-ized, the array or membrane filter used by this invention may be produced based on a well-known approach, even if it is a commercial thing (for example, intelligent gene:TAKARA SHUZO [CO., LTD.] make, an atlas system: Clonetec make etc.). Cloning cDNA or the RT-PCR product which performed a reverse transcriptase reaction and PCR by the primer produced based on array information, such as GenBank, and was produced is used for the solid-phase-ized gene.
[0061]
In the detection using an array, in case (Pori A) +RNA to cDNA is produced at a reverse transcriptase reaction, an indicator probe is prepared by adding d-UTP by which the indicator was carried out by the fluorochrome (for example, Cy3, Cy5 grade). If the indicator of (Pori A) +RNA under the administration conditions of an examined substance and the (Pori A) +RNA under the conditions of an examined substance of not prescribing a medicine for the patient is carried out with coloring matter different, respectively at this time, at the time of next hybridization, both can be mixed and it can measure at once. If detection is the commercial array of for example, TAKARA SHUZO CO., LTD., according to the protocol of the company, it will perform hybridization and washing and will perform detection of a fluorescence signal, and analysis using fluorescence signal detection machines (for example, a GMS418 array scanner: TAKARA SHUZO CO., LTD. make etc.).

[0062]
In the detection using a membrane filter, in case (Pori A) +RNA to cDNA is produced at a reverse transcriptase reaction, by adding d-CPT by which the indicator was carried out with radioisotope (for example, 32P, 33P), an indicator probe is prepared and hybridization is performed with a conventional method. For example, the commercial microarray made from a filter: In the case of an atlas system (Clonetec make), analyze by detecting using analysis equipments (for example, an atlas image: Clonetec make etc.) after performing hybridization and washing.
[0063]
Also when using which solid phase-ized sample, both the probes of the sample (under administration of an examined substance and the condition of not prescribing a medicine for the patient) to compare are made to hybridize, respectively, and a difference of the amount of gene expression is detected. At this time, the hybridization conditions of each probe are made the same. A difference of the amount of gene expression is detectable by making one solid phase-ized sample hybridize the mixture of both probes at once, and reading fluorescence intensity, if the indicator of each probe is carried out by different fluorochrome in the case of the fluorescent-labeling probe as mentioned above (Brown, P.O.et al.(1999) Nature genet.21, suppliment, 33-37).
[0064]
ii) RT-PCR method (real-time PCR method)
RT-PCR method and its real time PCR (TaqMan PCR) whose number is one — law is suitable for the evaluation approach of this invention high sensitivity and in that it is quantitatively detectable in minute amount DNA.
[0065]
real time PCR (TaqMan PCR) — law — 5' edge — a fluorochrome (reporter) — 3' — the oligonucleotide probe which an indicator is carried out by the fluorochrome (quencher) and hybridizes an edge to the specific region of the purpose gene is used. As for this probe, a reporter's fluorescence is controlled by the quencher in the usual condition. In the condition of having made the purpose gene hybridizing this fluorescent probe completely, it is that outside to Taq. PCR is performed using DNA polymerase. Taq If the expanding reaction by DNA polymerase progresses, a fluorescent probe will be hydrolyzed by the exonuclease activity from 5' edge, reporter coloring matter will separate, and fluorescence will be emitted. The real-time PCR method can carry out the quantum of the primary quantity of template DNA correctly by carrying out monitoring of this fluorescence intensity on real time.
[0066]

For example, if it is the case of this invention, the probe (for example, probe which consists of a base sequence shown in the array numbers 33-40) for detecting specifically the primer (for example, primer which consists of a base sequence shown in the array numbers 17-32) which amplifies a target gene (mRNA) specifically, and a target gene will be designed, real time PCR (TaqMan PCR) will be performed, and the amount of target gene expression will be detected and analyzed.

[0067]

iii) The other analysis approaches

As an approach of analyzing the amount of gene expression in addition to the above For example Subtraction technique () [Sive,] [H.L.] and John and T.St. (1988) Nucleic Acids Research 16, 10937, Wang, Z., and Brown, D.D.(1991) Proc.Natl.Acad.Sci.U.S.A.88, 11505-11509, The differential displaying method (it Liang(s)) P., and Pardee, and A.B. Science 257, (1992) 967-971, Liang, P., Averboukh, L., Keyomarsi, K., Sager, R., and Pardee, A.B.(1992) Cancer Research 52, 6966-6968, A differential hybridization method (John and T.St., and Davis, R.W.Cell (1979) 16, 443-452), moreover A suitable probe The used cross hybridization method () ["Molecular Cloning and A Laboratory Manual" Maniatis, T., Fritsch, E.F., Sambrook,] [J.] (1989) Cold Spring Harbor Laboratory Press etc. can be mentioned. The above-mentioned approach is useful when carrying out evaluation examination of two or more target gene expression profiles on the whole.

[0068]

a) Subtraction croning process :

A subtraction croning process is the approach of carrying out cloning of the gene by acquiring cDNA of the gene specifically discovered into a specific cell, and screening a cDNA library by using this cDNA as a probe. As the approach of subtraction, a single strand cDNA is produced from all RNA. After making all RNA obtained from the cell different from this hybridize, the single stranded DNA which was not hybridized in a hydroxyapatite column is isolated. the approach (the biotechnology manual series 3 and a gene-cloning laboratory procedure --) of producing the cDNA library from this cDNA Yodosha (1993) Current PUROTO call Inn molecular biology, Produce a cDNA library first and a single stranded DNA is prepared using a helper phage etc. from this library. After making what carried out the biotin indicator to all RNA obtained from the cell different from this single stranded DNA hybridize, The single stranded DNA which was not hybridized using avidin is isolated. By DNA polymerase It returns to a double strand. A cDNA library How to produce () [Tanaka, H., Yoshimura, Y., Nishina, Y., Nozaki, M., Nojima, H., and Nishimune] [Y.(1994) FEBS] Lett.355, 4-10, etc. are mentioned.

[0069]

cDNA is compounded with reverse transcriptase by using as mold all RNA that refined mRNA or all RNA first about administration of an examined substance, or each specimen under the conditions of not prescribing a medicine for the patient, and was specifically refined from the specimen under administration conditions. The indicator of the cDNA can also be carried out by adding [alpha-32P] dNTP at the time of composition. Although all RNA used as cDNA by which the indicator was carried out, and mold forms the stable double-stranded-DNA-RNA hybrid, only RNA is decomposed and a single strand cDNA is made to generate by carrying out high temperature processing under alkali existence. If this single strand cDNA and RNA extracted from the specimen under the conditions of not prescribing a medicine for the patient are mixed and it puts under suitable conditions, a stable double-stranded-DNA-RNA hybrid will be formed from the complementarity of a nucleotide sequence. That is, although cDNA which uses as mold all RNA discovered also under the conditions of not prescribing a medicine for the patient forms a hybrid, cDNA which used as mold RNA specifically discovered only under administration conditions is still a single strand. Subsequently, a hydroxyapatite column separates a double-stranded-DNA-RNA hybrid and a single strand cDNA, and a single strand cDNA is refined. Specific cDNA can be condensed by repeating this step in the organization which considered as the purpose. Condensed specific cDNA can be used as a probe which screens a cDNA library, when the indicator is carried out with radioisotope etc. In addition, this actuation can also be performed using commercial kits (for example, an PCR selection cDNA subtraction kit: Clonetec make etc.).

[0070]
b) The differential displaying method :
According to Liang's and others approach (Science (1992) 257, 967-971), the differential displaying method is the following, and can be made and enforced. First, mRNA or all RNA is extracted from two samples (in the case of this invention, they are administration of an examined substance, and a specimen under the conditions of not prescribing a medicine for the patient) to compare, and this is changed into a single strand cDNA using reverse transcriptase. Subsequently, PCR is performed by using the obtained single strand cDNA as mold using a suitable primer. As a primer, a random primer (primer of about ten to 12 mer which consists of an array of arbitration) can be used, for example. Or you may use combining support Doppler IMA (anchored primer) and every one sort each of ABITO rally primers (arbitrary primer). as support Doppler IMA -- oligo d (T) -- the primer which consists of nVX [n= 11 - a 12;V= guanine, an adenine or a cytosine;X= guanine, an adenine, a thymine, or a cytosine] can be used. Moreover, as an ABITO rally primer, the random primer of about 10 mer(s) which consists of an array of arbitration can be used. It becomes possible to screen a wide range gene cluster by performing such PCR combining various primers.
[0071]
Then, the gene specifically discovered by one of specimens can be isolated by carrying out gel electrophoresis of the acquired PCR product, and carrying out comparison analysis of the manifestation pattern (fingerprints) of all RNA developed on gel (display). In addition, this approach can also be performed using the kits (for example, an RNA image kit: JIEN hunter company make etc.) marketed.
[0072]
c) Differential hybridization method :
A differential hybridization method screens the cDNA library produced from all RNA of the target organization with the 32P indicator cDNA probe compounded from all RNA of the purpose organization and a contrast organization, and are the probe of the purpose organization, and the approach of choosing the clone which hybridizes. For example, from all RNA first refined from the specimen under the conditions of an examined substance of not prescribing a medicine for the patient, a cDNA library is produced according to a conventional method, and 2 sets of replica filters are produced from the library. Next, cDNA is compounded with reverse transcriptase by using as mold all RNA refined from the specimen under these conditions of not prescribing a medicine for the patient. The indicator of the cDNA is carried out by adding [alpha-32P] dNTP at the time of composition. Although all RNA used as cDNA by which the indicator was carried out, and mold forms the stable double-stranded-DNA-RNA hybrid, by carrying out high temperature processing under alkali existence, only all RNA can be decomposed and a single strand cDNA can be refined. The single strand cDNA by which similarly the indicator was carried out by 32P by using as mold all RNA refined from the specimen under the administration conditions of an examined substance is produced. The filter and hybridization which were produced from the specimen under the conditions of not prescribing a medicine for the patient are performed by using both indicator cDNA of these as a probe, respectively. The clone which hybridizes the autoradiography image of an X-ray film as compared with the last only to one side of the cDNA probe under administration or the conditions of not prescribing a medicine for the patient is chosen. In this way, cloning of the gene from which the amount of manifestations changes with administration of an examined substance specifically can be carried out.
[0073]
d) Cross hybridization method :
A cross hybridization method is the approach of choosing the clone which performed hybridization on the conditions that stringency is low, by having used suitable DNA as the probe to the cDNA library originating in one under administration of an examined substance, or the conditions of not prescribing a medicine for the patient of specimens, and has been discovered only to one side. That is, an electropositive clone is obtained by said hybridization, Northern hybridization is performed to all RNA originating in each specimen by using this electropositive clone as a probe, and the clone discovered only to one side is chosen.

[0074]
In this way, it can check that the selected gene is specifically discovered under administration conditions by performing Northern blotting by using obtained cDNA as a probe to all RNA of the specimen under administration or the conditions of not prescribing a medicine for the patient.
[0075]
Process 3: Evaluation of a beta cell malfunction improvement effect
At the last, the beta cell malfunction improvement effect of this examined substance is evaluated based on administration of an examined substance, and a difference of the amount of target gene expression under the conditions of not prescribing a medicine for the patient.
[0076]
That is, when the amount of target gene expression is decreasing intentionally under the administration conditions of an examined substance rather than the bottom of the condition of not prescribing a medicine for the patient, it can be estimated that this examined substance has a beta cell malfunction improvement effect. Here, it means that statistical significance ($p < 0.05$) is in the amount of target gene expression under administration of an examined substance, and the conditions of not prescribing a medicine for the patient, saying "it is decreasing intentionally."
[0077]
In addition, evaluation may carry out independent evaluation of the one amount of gene expression chosen from eight target genes, and may evaluate the two or more amounts of gene expression (manifestation profile) chosen from eight target genes on the whole.
[0078]
2.2 Evaluation Approach of Examined Substance Which Made Manifestation of Product of Target Gene Index (Inch Vivo)
As for evaluation of the beta cell malfunction improvement effect of the examined substance in in vivo which made the index the amount of manifestations of the protein (array numbers 9-16) which is the product of a target gene, it is desirable that it is an approach including the following process.

Process 1: Breed an animal under the conditions of administration of an examined substance, or not prescribing a medicine for the patient.
Process 2: Detect the protein shown according to any one amino acid sequence of the blood of the above-mentioned animal, or the array numbers 9-16 in a cell using the antibody specifically combined with this protein.
Process 3: Evaluate the beta cell malfunction improvement effect of this examined substance based on a difference of the amount of manifestations of the above-mentioned protein in administration and un-prescribing a medicine for the patient. [of an examined substance]
[0079]
Process 1: Breeding of an animal
An animal is bred according to the approach indicated for the preceding clause 2.1 under the conditions of administration of an examined substance, or not prescribing a medicine for the patient.
[0080]
Process 2: Detection of the amount of manifestations of protein
Next, from the animal bred under administration of an examined substance, or the conditions of not prescribing a medicine for the patient, blood or a cell is isolated and the amount of manifestations of the protein shown according to any one amino acid sequence of the array numbers 9-16 in this blood or a cell is detected using the antibody specifically combined with this protein.
[0081]
(1) Preparation of a sample
The cell of a specimen which the product of blood or a target gene has high-discovered is desirable, therefore a pancreas Langerhans Mr. islet cell, especially its beta cell are desirable. After removing the insoluble matter by performing high-speed centrifugal one if needed, as said blood or cell (it is used as a cell extract) is the following, it is prepared as a sample for detection.
[0082]

What was suitably diluted with the buffer solution, using the collected blood serum as it is is used for the sample for solid phase enzyme immunoassay (the ELISA method) or radioisotope immunoassay (the RIA method). The sample for western blotting (for electrophoresis) is suitably diluted with the buffer solution, using a cell extract as it is, and what was mixed with the sample buffer solutions (sigma company make etc.) containing 2-Mercator ethanol for SDS-polyacrylamide gel electrophoreses is used. The thing which the collected cell extract itself or the thing suitably diluted with the buffer solution was used [thing], and made it adsorb blotting equipment to a direct membrane for example is used for a dot / sample for slot blotting methods.

[0083]

(2) Solid-phase-izing of a sample

In detection of the protein using an antibody, the polypeptide in the sample in which the protein which should be detected is contained first is solid-phase-ized on the base in a well of a membrane or 96 hole plate etc.

[0084]

In western blotting, and a dot / slot blotting methods, a sample is solid-phase-ized to a membrane. Once solid phase-ization carries out polyacrylamide gel electrophoresis of the sample, it can mention the approach (western blotting) of imprinting the developed polypeptide to a membrane, and the method (a dot / slot blotting methods) of infiltrating a sample or its diluent into a direct membrane. As a membrane used, nitrocellulose membranes (for example, Bio-Rad make etc.), nylon membranes (for example, yes bond-ECL (Amersham Pharmacia manufacture) etc.), cotton membranes (for example, blot ABUSO vent filter (Bio-Rad make) etc.), or the poly vinylidene JIFURUORIDO (PVDF) membranes (for example, Bio-Rad make etc.) can be mentioned. Moreover, as the blotting approach, the wet type blotting method (CURRENT PROTOCOLS IN IMMUNOLOGY volume 2 ed by J.E.Coligan, A.M.Kruisbeek, D.H.Margulies, E.M.Shevach, W.Strober), the semi dry type blotting method (above-mentioned CURRENT PROTOCOLS IN IMMUNOLOGY volume 2 reference), etc. can be mentioned.

[0085]

On the other hand, by the ELISA method or the RIA method, a sample is solid-phase-ized on 96 hole plate. solid-phase-izing -- for example, said 96 hole plates (for example, immuno plate maxi soap (Nunc make) etc.) -- a sample or its diluent (for example, thing diluted with the phosphate buffered saline (henceforth "PBS") containing 0.05% sodium azide) -- putting in -- 4 degrees C - a room temperature -- a night or 37 degrees C -- 1 - 3 hours -- putting -- a well -- what is necessary is just to make a polypeptide stick to a base

[0086]

(3) Preparation of an antibody

The antibody used at this process can carry out immunity of the animal using the polypeptide of the protein which should be detected, or the arbitration chosen from the amino acid sequence with a conventional method (397 for example, the new chemistry experiment lecture 1, protein 1, p.389-1992), and can obtain it by extracting and refining the antibody produced in this animal living body. Moreover, according to a well-known approach (for example, Kohler and Milstein, Nature 256, 495-497, 1975, Kennet, and R.ed., Monoclonal Antibody p.365-367, 1980, Prenum Press, N.Y.), the antibody forming cell which produces the target antibody may be united with a myeloma cell, a hybridoma may be established, and the monoclonal antibody obtained from this hybridoma may be used.

[0087]

The polypeptide which consists of its at least six continuous partial amino acid sequences as an antigen for antibody production, or the derivative which added the amino acid sequence and support of arbitration to these can be used. What combined keyhole limpet hemocyanin with the amino terminal of the protein which should be detected especially as support is desirable.

[0088]

A suitable host cell may be made to produce said antigen polypeptide using genetic manipulation. For example, what is necessary is to produce the vector in which the target gene expression of this invention is possible, to introduce this into a host cell, and just to make this gene discover.

[0089]
As said host cell, if it is a prokaryotic cell, Escherichia coli (Escherichia coli), a Bacillus subtilis (Bacillus subtilis), etc. will be mentioned, for example. In order to carry out the transformation of the target gene by these host intracellular, the transformation of the host cell is carried out by the plasmid vector containing a regulatory sequence, the replicon of the seed origin, i.e., the replication origin, which may suit with a host. What has the array which can give the selectivity of the quality of a phenotype (phenotype) to a transformed cell as this vector is desirable.
[0090]
For example, although K12 share etc. will be used well and the plasmid of a pBR322 and pUC system will generally be used as a vector if it is Escherichia coli, it is not limited to these but various well-known strain and a well-known vector can be used. Moreover, as a promotor used with Escherichia coli, a tryptophan (trp) promotor, a lactose (lac) promotor, a tryptophan lactose (tac) promotor, a lipoprotein (lpp) promotor, a polypeptide chain elongation factor Tu (tufB) promotor, etc. can be mentioned, and all can be used suitably, for example.
[0091]
Moreover, although 207 to 25 shares will be desirable and pTUB228 (Ohmura, K.et al.(1984) J.Biochem.95, 87-93) etc. will be used as a vector if it is a Bacillus subtilis, it is not limited to this. In addition, a secretion manifestation out of a fungus body is also attained by connecting the DNA array which carries out the code of the transit peptide array of the alpha-amylase of a Bacillus subtilis to a vector.
[0092]
Cells, such as a vertebrate, an insect, and yeast, are mentioned as a host cell of an eukaryotic cell. the COS cell (Gluzman, Y.(1981) Cell 23, 175-182, ATCC CRL-1650) which is a cell of an ape as a vertebrate cell, for example, and a Chinese hamster ovary cell (a CHO cell --) ATCC Although the dihydroleaf redox enzyme defect stock (Urlaub, G.and Chasin, L.A.(1980) Proc.Natl.Acad.Sci.USA 77, 4126-4220) of CCL-61 etc. is used well It is not limited to these.
[0093]
What has the promotor located in the upstream of the gene which you are going to make it usually discover as an expression vector of a vertebrate cell, the splice site of RNA, a polyadenylation part, a conclusion array of an imprint, etc. can be used. Furthermore, this may have a replication origin as occasion demands. Although pSV2dhfr (Subramani, S.et al.(1981) Mol.Cell.Biol.1, 854-864) which has pCR3.1 (product made from Invitrogen) and the initial promotor of SV40 who have the initial promotor of a cytomegalovirus is mentioned as an example of this expression vector, it is not limited to these.
[0094]
If the case where a COS cell is used is mentioned as an example as a host cell, as an expression vector, it has an SV40 replication origin, and in a COS cell, independence growth is possible, and a transcriptional promoter, the conclusion signal of an imprint, and the thing equipped with the RNA splice site can be used further suitably. This expression vector The diethylaminoethyl (DEAE)-dextran method (Luthman, H.and Magnusson, G.(1983) Nucleic Acids Res, 11, 1295-1308), A calcium phosphate-DNA coprecipitation Mr. method (Graham, F.L.and van der Eb, A.J.(1973) Virology 52, 456-457), The electric pulse terebration (Neumann, E.et al.(1982) EMBO J.1, 841-845), And the RIPOFE cushion method () [Lopata] By et al.(1984) Nucl.Acids Res.12, 5707-5717, Sussman and Milman (1984) Mol.Cell.Biol.4, 1641-1643, etc. A COS cell can be made to be able to incorporate and a desired transformed cell can be obtained in this way.
[0095]
Moreover, in using a CHO cell as a host cell, in order to choose a desired transformed cell, it is desirable to carry out coincidence transfection of this selective marker that connected and carried out transfection of the selective markers (for example, neomycin (or G418) resistance gene neo etc.), such as antibiotic resistance, to the introductory gene, or was prepared separately, and the introductory gene. The cell by which the transformation was carried out stably can be chosen by using the property of this selective marker after that.
[0096]

In using an insect cell as a host cell The ovarian cell origin established cell line (Sf-9 or Sf-21) of Spodoptera frugiperda of Lepidoptera Phalaenidae, and the ootid origin High Five cell of Trichoplusia ni () [Wickham,] [T.J.] et al (1992), Biotechnol.Prog.i: 391-396 etc. is well used as a host cell. As a baculovirus transfer vector pVL 1392/1393 using the promotor of the PORIHE drine compounds protein of a ** Autographa nuclear polyhedrosis virus (AcNPV) is used well (). [Kidd,] [i.M.and V.C.Emery (1993) The] use of baculoviruses as expression vectors.Applied Biochemistry and Biotechnology 420, 137-159. In addition, the vector using the promotor of the P10 and this basic protein of a baculovirus can also be used. Furthermore, it is possible by tying the secretion signal array of the envelope surface protein GP67 of AcNPV to the amino terminal side of the purpose protein to also make recombination protein discover as secretory protein (Zhe-mei Wang, et al.(1998) Biol.Chem., 379, 167-174).

[0097]

As a manifestation system which made the eukaryon microorganism the host cell, generally yeast is known well and Saccharomyces yeast, for example, baker's yeast Saccharomyces cerevisiae and petroleum yeast Pichia pastoris, is desirable also in it. As an expression vector of eukaryon microorganisms, such as this yeast For example The promotor of an alcoholic dehydrogenase gene () [Bennetzen,] [J.L.and] Hall and B.D.(1982) J. Biol.Chem.257, 3018-3025, the promotor (Miyanohara, A.et al.(1983) Proc.Natl.Acad.Sci.USA 80, 1-5) of an acid phosphatase gene, etc. It can use preferably. Moreover, when making it discovered as secretor protein, it is possible to also make it discovered as recombinant which has at least the cutting section of the internality protease or the known protease which a secretion signal array and a host cell have in an amino terminal side. for example, by the system made to discover with petroleum yeast, Homo sapiens mast cell TORIPUTAZE of a trypsin mold serine protease By carrying out the bond manifestation only of the cutting section of KEX2 protease which the secretion signal array of alpha factor of yeast and petroleum yeast have in an amino terminal side It is known that active TORIPUTAZE will be secreted in a culture medium (Andrew, L.Niles, et al.(1998) Biotechnol.Appl.Biochem.28, 125-131).

[0098]

The transformant obtained as mentioned above produces the target protein out of intracellular [the] or a cell by cultivating according to a conventional method. Various kinds of culture media commonly used as a culture medium used for this culture according to the adopted host cell can be chosen suitably. For example, if it is the above-mentioned COS cell, what added blood serum components, such as fetal calf serum, if needed to culture media, such as RPMI1640 culture medium and an Dulbecco's modified Eagle's medium (henceforth "DMEM"), can be used.

[0099]

It can dissociate and refine by the well-known separation [which was produced out of intracellular / of a transformant /, or a cell by the above-mentioned culture] operation information for which protein used the physical property, chemical property, etc. by rearranging. independent [in various liquid chromatography, such as processing according to a protein precipitant for example, an ultrafiltration, a molecular sieve chromatography (gel filtration), adsorption chromatography, an ion exchange chromatography, affinity chromatography, and high performance chromatography (HPLC), and dialysis] as such an approach -- or it can combine and use. Moreover, if the histidine which becomes the recombination protein made to discover from 6 residue is connected, it can also refine efficiently by the nickel affinity column. The target protein can be easily manufactured by high yield and the high grade by combining suitably the approach indicated above.

[0100]

(4) Detection

The obtained antibody makes independence or this antibody a primary antibody, and is used for detection combining the indicator (antibody of the animal origin which produced antibody is recognized) second antibody which recognizes this specifically.

[0101]

Although a thing desirable as a class of said indicator is an enzyme (alkaline phosphatase or horseradish peroxidase) or a biotin (however, actuation of combining enzyme-labeling streptoavidin

with the biotin of a second antibody further is added), it is not limited to these. As an indicator second antibody (or indicator streptoavidin), various marketing of the antibody (or streptoavidin) by which the indicator was carried out beforehand is carried out. In addition, measurement is performed using a liquid scintillation counter etc. using the antibody to which the indicator of the case of RIA was carried out with radioisotope, such as 125I.

[0102]

The amount of manifestations of the protein which should be detected is measured by detecting the activity of these enzymes by which the indicator was carried out. In addition, when carrying out an indicator by the alkaline phosphatase or horseradish peroxidase, the substrate colored according to the catalyst of these enzymes and the substrate which emits light are marketed.

[0103]

When the substrate to color is used, if western blotting, and a dot / slot blotting methods are used, it can detect visually. It is desirable to measure and carry out the quantum of the absorbance (for measurement wavelength to change with substrates) of each well by the ELISA method using a commercial microplate reader. Moreover, it is also possible to carry out the quantum of the antigen concentration in other samples by preparing the dilution sequence of the antigen used for above-mentioned antibody production, performing detection actuation to other samples and coincidence by making this into a standard antigen sample, and creating the standard curve which plotted standard antigen concentration and measured value.

[0104]

On the other hand, when the substrate which emits light is used, in western blotting, or a dot / slot blotting methods, the autoradiography using an X-ray film or an imaging plate and the photography using an instant camera can detect. Moreover, the quantum using a densitometry, a molecular imager Fx system (Bio-Rad make), etc. is also possible. Furthermore, when using a luminescence substrate by the ELISA method, enzyme activity is measured using luminescence microplate readers (for example, Bio-Rad make etc.).

[0105]

(5) Measurement actuation

a) In the case of western blotting, a dot blot, or a slot blot

First, in order to prevent the nonspecific adsorption of an antibody, actuation (blocking) dipped fixed time into the buffer solution containing the matter (skim milk, casein, bovine serum albumin, gelatin, polyvinyl pyrrolidone, etc.) which checks such nonspecific adsorption for a membrane beforehand is performed. The presentation of a blocking solution is 5%. Skim milk, 0.05 - 0.1% The phosphate buffered saline (PBS) or the tris buffered saline solution (TBS) containing Tween 20 is used. Instead of skim milk, the block ace (Dainippon Pharmaceutical), 1 - 10% of bovine serum albumin, 0.5 - 3% of gelatin, or 1% of polyvinyl pyrrolidone may be used. The time amount of blocking is 1 - 3 hours at 16 - 24 hours, or a room temperature in 4 degrees C.

[0106]

Next, it is a membrane 0.05 - 0.1% After washing in PBS or TBS (henceforth a "penetrant remover") containing Tween 20 and removing an excessive blocking solution, an antibody is dipped fixed time into the solution suitably diluted with the blocking solution, and this antibody is combined with the antigen on a membrane. The dilution scale factor of the antibody at this time can be determined by conducting the preliminary Western-blotting experiment which made the sample what carried out phase dilution of said recombination antigen, for example. This antibody reaction actuation is preferably performed at a room temperature for 2 hours. A membrane is washed by the penetrant remover after antibody reaction actuation termination. Here, when the indicator of the used antibody is carried out, detection actuation can be performed immediately. When the antibody of a non-indicator is used, a second antibody reaction is performed succeedingly. An indicator second antibody is diluted and used 2000 to 20000 times with a blocking solution, when using a commercial thing (the publication is followed when the suitable dilution scale factor for attached instructions is indicated). After dipping the membrane after carrying out washing removal of the primary antibody in a second antibody solution at a room temperature for 45 minutes to 1 hour and washing by the penetrant remover, detection actuation doubled with the

indicator approach is performed. Washing actuation is performed by exchanging penetrant removers again and shaking for 5 minutes, after shaking a membrane for 15 minutes in a penetrant remover first, exchanging a penetrant remover for a new thing and shaking it for 5 minutes for example. A penetrant remover may be exchanged and washed further if needed.

[0107]

b) The ELISA method / the RIA method

First, in order to prevent the nonspecific adsorption of the antibody to the base in a well of the plate which made the sample solid-phase-ize, it blocks beforehand like the case of western blotting. It is as having indicated the conditions of blocking in the term of western blotting.

[0108]

Next, it is the inside of a well 0.05 - 0.1% After washing in PBS or TBS (henceforth a "penetrant remover") containing Tween 20 and removing an excessive blocking solution, a fixed time amount incubation of the antibody suitably diluted with the penetrant remover is poured distributively and carried out, and this antibody is combined with an antigen. The dilution scale factor of the antibody at this time can be determined by conducting the preliminary ELISA experiment which made the sample what carried out phase dilution for example, of the above-mentioned recombination antigen. This antibody reaction actuation is preferably performed at a room temperature for about 1 hour. After antibody reaction actuation termination and the inside of a well are washed by the penetrant remover. Here, when the indicator of the used antibody is carried out, detection actuation can be performed immediately. When the antibody of a non-indicator is used, a second antibody reaction is performed succeedingly. An indicator second antibody is diluted and used 2000 to 20000 times by the penetrant remover, when using a commercial thing (the publication is followed when the suitable dilution scale factor for attached instructions is indicated). After pouring a second antibody solution distributively to the well after carrying out washing removal of the primary antibody, carrying out an incubation at a room temperature for 1 to 3 hours and washing by the penetrant remover, detection actuation doubled with the indicator approach is performed. Washing actuation is performed by exchanging penetrant removers again and shaking for 5 minutes, after pouring a penetrant remover distributively, shaking for 5 minutes in a well first for example, exchanging a penetrant remover for a new thing and shaking it for 5 minutes. A penetrant remover may be exchanged and washed further if needed.

[0109]

For example, in this invention, the so-called ELISA of a sandwich technique can be carried out by the approach of indicating below. First, two fields which are rich in a hydrophilic property are chosen from each amino acid sequence of the protein which should be detected, respectively. Next, the partial peptide which consists of 6 or more residue of amino acid in each field is compounded, and two kinds of antibodies which used this partial peptide as the antigen are acquired. Among these, the indicator of one antibody is carried out. The antibody of the direction which did not carry out an indicator is solid-phase-ized on the base in a well of the plate for 96 hole ELISA. After blocking, a sample solution is paid in a well and an incubation is carried out in ordinary temperature for 1 hour. The incubation of the antibody diluent of the direction which carried out the indicator is poured distributively and carried out to each well after washing the inside of a well. Detection actuation doubled with the indicator approach is performed after washing the inside of a well again.

[0110]

Process 3: Evaluation of a beta cell malfunction improvement effect

At the last, the beta cell malfunction improvement effect of this examined substance is evaluated based on the difference of the amount of manifestations of the protein shown according to any one amino acid sequence of the array numbers 9-16 in administration and un-prescribing a medicine for the patient. [of an examined substance]

[0111]

That is, when the amount of manifestations of said protein is decreasing intentionally under the administration conditions of an examined substance rather than the bottom of the condition of not prescribing a medicine for the patient, it can be estimated that this examined substance has a beta

cell malfunction improvement effect. Here, it means that statistical significance ($p < 0.05$) is in the amount of manifestations of administration of an examined substance, and this protein under the conditions of not prescribing a medicine for the patient, saying "it is decreasing intentionally."

[0112]

Independent evaluation of the amount of manifestations of any 1 protein may be carried out among eight sorts of proteins shown according to any one amino acid sequence of the array numbers 9-16, and the amount of manifestations of two or more proteins chosen from these (manifestation profile) may be evaluated on the whole.

[0113]

2.3 Evaluation Approach of Examined Substance Which Made Target Gene or Its Product Index (Inch Vitro)

As for the evaluation approach of the beta cell malfunction improvement effect of the examined substance in in vitro which made a target gene or its product the index, it is desirable to include the following process.

[0114]

Process 1: Cultivate a cell under addition of a specimen material, or the conditions of not adding.

Process 2: Detect the amount of target gene expression in the above-mentioned cell, or detect the amount of manifestations of the protein which is the product using the antibody specifically combined with this protein.

Process 3: Evaluate the beta cell malfunction improvement effect of this examined substance based on a difference of the amount of manifestations of the protein which is the above-mentioned target gene under addition of a specimen material and the conditions of not adding, or its product.

[0115]

Process 1: Culture of a cell

The cell used by the evaluation approach of this invention will not be limited especially if it is the mammalian cell which has discovered the target gene concerning this invention. The cultured cell of the mammalian origin, especially the Langerhans Mr. island origin of mammalian is preferably desirable, and especially the beta cell is desirable. As mammalian, Homo sapiens, a mouse, a rat, or a hamster is desirable, and Homo sapiens or a mouse is more desirable.

[0116]

Said especially cell has the desirable cell which has high-discovered the target gene concerning this invention reflecting the beta cell malfunction symptoms in diabetes mellitus. As such a cell, the primary culture cell of the type 2 diabetes model animal (for example, above-mentioned diabetes-mellitus model mouse etc.) origin can be mentioned, for example. Moreover, cells by which the transformation was carried out artificially, such as a cell which introduced the target gene of this invention with the promoterregion, may be produced and used.

[0117]

A cell is cultivated under addition of a specimen material, or the conditions of not adding. Especially the culture approach is not limited but should just choose the culture approach suitable for the cell concerned suitably. Neither the addition (administration) approach of the specimen material to a cultured cell nor especially an addition is also limited, for example, a specimen material is added to a culture culture medium, and carrying out fixed period culture etc. should just carry out a cell. Although what is necessary is just to also set up suitably the period cultivated under specimen material existence, it is 30 minutes - 24 hours preferably.

[0118]

Process 2: Detection of a target gene or the amount of manifestations of the product

Next, a difference of the amount of target gene expression in addition of a specimen material and the above-mentioned cell under the conditions of not adding is detected, or a difference of the amount of manifestations of the protein which is the product is detected using the antibody specifically combined with this protein.

[0119]

What is necessary is just to perform detection of a target gene according to the approach

fundamentally indicated to 2.1. Moreover, what is necessary is just to perform detection of the protein using an antibody according to the approach indicated to 2.2.

[0120]

The manifestation of a target gene or its product is also indirectly detectable under promotor rule of a target gene besides the above-mentioned approach using the gene (henceforth a "reporter gene") which enables detection of this promotor activity. Hereafter, the detection approach using a reporter gene is explained.

[0121]

(1) Reporter gene

A reporter gene should just carry out the code of the distinguishable reporter protein to any protein of the others which a host cell can produce in a series of processes of the exam approach clearly. That in which the cell before a transformation does not have the gene which carries out the code of the protein the same as that of this reporter protein or similar preferably is good. For example, even when it is that in which reporter protein has toxicity to this cell, and the thing which this cell gives the resistance of the antibiotic which has susceptibility, the existence of a manifestation of a reporter gene can be judged with the survival rate of a cell. However, the more desirable thing as a reporter gene used by this invention is the structural gene (as [acquire / for example, / the specific antibody to the protein by which a code is carried out to this reporter gene]) which can detect the amount of manifestations specifically and quantitatively. It is the gene which carries out the code of the enzyme with which quantitive measurement produces easy metabolite by reacting specifically with a foreign substrate more preferably. As such a reporter gene, although the gene which carries out the code of the following enzymes and proteins can be illustrated, this invention is not limited to them, for example.

[0122]

a) Chloramphenicol acetyltransferase :

It is detectable by the so-called CAT assay etc. with the enzyme which adds an acetyl group to a chloramphenicol. As a vector which can prepare the vector for reporter assays only by incorporating a promotor, the pCAT3-Basic vector (pro megger company make) is marketed.

b) Firefly luciferase :

A quantum can be carried out by measuring the bioluminescence produced when luciferin is metabolized. As a vector for reporter assays, the pGL3-Basic vector (pro megger company make) is marketed.

c) Beta-galactosidase :

There is a respectively measurable substrate by color reaction, fluorescence, or chemiluminescence. As a vector for reporter assays, pbetagal-Basic (pro megger company make) is marketed.

d) Secretor alkaline phosphatase :

There is a respectively measurable substrate by color reaction, the bioluminescence, or chemiluminescence. pSEAP2-Basic (Clontech make) is marketed as a vector for reporter assays.

e) Green fluorescence protein (green-fluorescent protein) :

Although it is not an enzyme, since oneself emits fluorescence, a direct quantum can be carried out. pEGFP-1 (Clontech make) is marketed as same vector for reporter assays.

[0123]

(2) Installation of a reporter gene

According to a well-known approach, installation of a reporter gene produces the recombination vector which enabled the manifestation of a reporter gene manifestation plasmid and the target gene of this invention in the mammals cell, and should just carry out coincidence transfection of these to a cell. As a vector, although pCR3.1 (product made from in vitro JIEN), pCMV-Script (Stratagene make), etc. can be used suitably, it is not limited to these.

[0124]

As an approach of introducing a manifestation plasmid into a cell The DEAE-dextran method (Luthman, H.and Magnusson, G.(1983) Nucleic Acids Res.11, 1295-1308), A calcium phosphate-DNA coprecipitation Mr. method (Graham, F.L.and van der Eb, A.J.(1973) Virology 52,

456-457), The electric pulse terebration (Neumann, E.et al.(1982) EMBO J.1, 841-845), The RIPOFE cushion method () [Lopata et] Although al.(1984) Nucl.Acids Res.12, 5707-5717, Sussman and Milman (1984) Mol.Cell.Biol.4, 1641-1643, etc. can be mentioned It is not limited to these but the approach of the arbitration used widely can be adopted. However, when a cell is the so-called suspension cell, it is desirable to use approaches other than a calcium phosphate-DNA coprecipitation Mr. method. Also in which approach, it is required to use the optimum-ized transfection conditions according to the cell to be used.

[0125]

(3) Evaluation

If the cell which carried out coincidence transfection of the target gene expression vector and reporter expression vector of obtained this invention is cultivated, the imprint of a reporter gene will be promoted by this target gene expression dependence target. Therefore, if manifestation change of the reporter gene in the case where it does not add with the case where the specimen material of arbitration is added in a culture medium under the condition which can discover a reporter gene is seen, target gene expression change can be evaluated. Here, the cell transfected by the reporter expression vector survives and "the conditions which can discover a reporter gene" should just be conditions which can produce the product (reporter protein) of a reporter gene. It is the culture medium (blood serum components, such as fetal calf serum, may be added) which suited the cell strain used preferably, and culture is carried out for two - three days (most suitably for two days) at 34-40 degrees C (most suitably 37 degrees C) under the air existence containing 4 - 6% (most suitably 5%) of carbon dioxide gas.

[0126]

(4) In addition to this (establishment of a transformed cell stock)

The test method using the cell which are two expression vectors containing a reporter gene or the target gene of this invention, and carried out the transformation of the host cell to the duplex apart from the test method using the above passing away transgenics methods is also adoptable. In this case, it is necessary to establish a cell strain by which the manifestation of this reporter gene is promoted under the conditions which guide the target gene expression of this invention using expression vectors, such as pIND (product made from in vitro JIEN), and pTet-On (Clontech make). Being held stably is desirable, even if the gene introduced in such a transformed cell is included in the chromosome of a host cell and piles up the passage of a host cell. In order to choose a desired transformed cell as a host cell, it is desirable to carry out coincidence transfection of this selective marker that connected and carried out transfection of the selective markers (for example, neomycin (or G418) resistance gene neo etc.), such as antibiotic resistance, to the introductory gene, or was prepared separately, and the introductory gene. The cell by which the transformation was carried out stably can be chosen by using the property of this selective marker after that.

[0127]

In this way, in the obtained cell strain, the imprint of a reporter gene is promoted by this gene expression dependence target also under the condition to which the target gene expression of this invention is guided. Therefore, if the amount change of manifestations of the reporter gene in the case where it does not add with the case where a specimen material is added under the condition which can discover a reporter gene is seen, the amount of target gene expression can be evaluated.

[0128]

Process 3: Evaluation of a beta cell malfunction improvement effect

At the last, the beta cell malfunction improvement effect of this examined substance is evaluated based on a difference of the target gene in administration and un-prescribing a medicine for the patient or the amount of manifestations of the product. [of an examined substance]

[0129]

That is, when the target gene or the amount of manifestations of the product is decreasing intentionally under the administration conditions of an examined substance rather than the bottom of the condition of not prescribing a medicine for the patient, it can be estimated that this

examined substance has a beta cell malfunction improvement effect. Here, it means that statistical significance ($p < 0.05$) is in administration of an examined substance and the target gene under the conditions of not prescribing a medicine for the patient, or the amount of manifestations of the product, saying "it is decreasing intentionally."

[0130]

In addition, evaluation may carry out independent evaluation of one gene chosen from eight target genes, or the amount of manifestations of the product, and may evaluate two or more genes chosen from eight target genes, or the amount of manifestations of those products (manifestation profile) on the whole.

[0131]

3. Evaluation of Beta Cell Malfunction

The manifestation of the gene product (protein shown according to any one amino acid sequence of the array numbers 9-16) shown according to any one base sequence of the array numbers 1-8 concerning this invention serves as an index of a beta cell malfunction. Therefore, a test subject's beta cell malfunction (diabetic symptoms) can be diagnosed by making into an index the amount of manifestations of the HITOOSOROGU product (array numbers 57-64) of the gene shown according to any one base sequence of the array numbers 1-8 in blood.

[0132]

That is, this invention offers the approach of evaluating this test subject's beta cell malfunction, by measuring the HITOOSOROGU product of manifestations of the gene shown according to any one base sequence of the array numbers 1-8 in the blood (specimen) isolated from the test subject, i.e., the amount of the protein shown according to any one amino acid sequence of the array numbers 57-64.

[0133]

Measurement of the amount of manifestations of the protein in a specimen can be carried out according to the process 2 of the preceding clause 2.2. The amount of manifestations of the protein concerned in a specimen can estimate this test subject that possibility of having produced a beta cell malfunction is high, when intentionally high as compared with forward Tsuneto. It means that statistical significance ($p < 0.05$) is in the amount of manifestations of the this ["be / it / intentionally high"] protein in the blood of healthy people and a test subject here.

[0134]

The above-mentioned evaluation may carry out independent evaluation of the amount of manifestations of any 1 protein among the proteins shown according to any one amino acid sequence of the array numbers 57-64, and may evaluate the amount of manifestations of two or more proteins chosen from these (manifestation profile) on the whole.

[0135]

4. Kit for Evaluation of Beta Cell Malfunction Improvement Effect

This invention offers the kit for evaluation of the beta cell malfunction improvement effect an examined substance's which made the index the target gene this invention's, or the amount of manifestations the product's, or a test subject's beta cell malfunction again.
 [0136]

Said kit contains at least one or more [which is chosen from the group which consists of the following a-e].

a) The oligonucleotide primer which 15 - 30 base length for amplifying specifically the gene shown according to any one base sequence of the array numbers 1-8 followed

b) The polynucleotide probe with which the 20 - 1500 base length for combining with the gene shown according to any one base sequence of the array numbers 1-8 specifically, and detecting this gene continued

c) The solid phase-ized sample to which the polynucleotide probe given [above-mentioned] in b was fixed

d) The antibody for combining with the protein shown according to any one amino acid sequence of the array numbers 9-16 specifically, and detecting this protein

e) The second antibody which can be specifically combined with an antibody given [above-mentioned] in d

[0137]
Based on the base sequence (array numbers 1-8) of the target gene of this invention, according to a conventional method, it can design using commercial primer design software etc. easily, and a primer given [said] in a can amplify it. The oligonucleotide which has the base sequence shown, for example in the array numbers 17-32 as an example of such a primer can be mentioned.
[0138]
Moreover, a probe given [said] in b is a polynucleotide specifically hybridized in the target gene (array numbers 1-8) of this invention, and its thing of 20 - 1500 base length extent is desirable. If it is the Northern hybridization method, specifically, the single-strand oligonucleotide or the double stranded DNA of 20 base length extent will be used suitably. Moreover, if it is a microarray, the double stranded DNA of 100 - 1500 base length extent or the single-strand oligonucleotide of 20 - 100 base length extent will be used suitably. On the other hand, the GeneChipTM system of Affimetrix has good single-strand oligo of 25 base length extent. As for especially these, it is desirable to design as a probe which the array singularity which exists in 3' untranslation region of the target gene of this invention hybridizes specifically into a high part. The label of for example, enzyme labeling, a radioactive indicator, fluorescent labeling, etc. may be carried out with the suitable indicator reagent, and these primers and probes may be embellished by the biotin, the phosphoric acid, the amine, etc. The oligonucleotide which has the base sequence shown in the array numbers 33-40 as an example of such a probe can be mentioned.
[0139]
Moreover, a solid phase-ized sample given [said] in c is produced by fixing a probe given [said] in b to the solid phase of a glass plate, a nylon membrane, a micro bead, a silicon chip, etc. Although 2.1 already explained such a solid phase-ized sample and its production approach, a gene chip, a cDNA array, an oligo array, a membrane filter, etc. can be mentioned, for example.
[0140]
An antibody said d and given in e is producible by the approach indicated to 2.2. The label of for example, enzyme labeling, a radioactive indicator, fluorescent labeling, etc. may be carried out with the suitable indicator reagent, and this antibody may be suitably embellished by the biotin etc.
[0141]
The kit of this invention may include detection of hybridization, the indicator of a probe, and a label object etc. for the reagent of an and also [it is the need] etc. in the evaluation approach concerning this invention suitably if needed besides the above-mentioned component.
[0142]
5. In Addition to this
5.1 Prediction of Beta Cell Malfunction
The target gene concerning this invention is a gene high-discovered by the type 2 diabetes model animal. Therefore, this amount of gene expression increases reflecting the symptoms of the type 2 diabetes in Homo sapiens. Since a manifestation is normalized by improvement of a beta cell malfunction, especially these genes are considered reflecting the beta cell malfunction in Homo sapiens. It is possible to detect change of the minute amount amount of manifestations at an early stage rather than the manifestation of the protein which is the gene product concerned with the gene which the amplifying methods, such as PCR, have established.
[0143]
It is also possible by following, for example, measuring the amount of manifestations of the gene (array numbers 49-56) concerned in a test subject's blood and cell to predict the symptoms of this test subject's diabetes mellitus, especially the improvement of beta cell malfunction **. Such prediction can be more correctly performed by analyzing gene expression profiles, such as the factor which reflects other beta cell malfunctions with a target gene, for example, TNF-alpha, ADIPONE cutin, etc., on the whole.
[0144]
5.2 Production of Model Animal Which Has Beta Cell Malfunction
By raising artificially the manifestation of the target gene concerning this invention, or its product, it is also possible to produce the animals (for example, mouse etc.) which have a beta cell

malfunction. For example, the transgenic animal which high-discovers said target gene (array numbers 1-8) is produced, and if the beta cell malfunction symptoms represented by Homo sapiens type 2 diabetes appear, research on a beta cell malfunction or its improvement can be done using this animal. It is thought possible by similarly medicating a direct animal with the protein (array numbers 9-16) which are these products to produce the model animal which has a beta cell malfunction.

[Example]

[0145]

Hereafter, although an example and the example of reference explain this invention to a detail further, this invention is not limited to these examples.

[0146]

[Example 1] The medication to a C57 BL/KsJ db/db mouse, and blood biochemistry value measurement

1. Administration Drug

Insulin-resistance improvement agent: 5-[4-(6-methoxy-1-methylbenzimidazol-2-ylmethoxy) benzyl] thiazolidine -2, 4-dione hydrochloride (about the process of; compound A indicated to be "compound A", it is JP,9-295970,A (U.S. Pat. No. 5886014 specification, Europe patent application public presentation No. 745600 specification) and example of reference 1 reference hereafter)

[0147]

2. Animal

The male C57 BL/KsJ db/db mouse was used as a diabetes-mellitus model mouse, and the male C57 BL/KsJ db/+m mouse (both made in Japanese Clare) was used as a normal mouse. After acclimating a mouse for about one week after purchase by 5 weeks old, the experiment was presented with it by 6 weeks old. Drinking water and baiting (F2, made in the Funabashi farm) considered during habituation / administration period as free intake. Breeding and the experiment during an administration period were conducted in the laboratory animal area managed by the laboratory animal management office.

[0148]

3. Test Method

a) Treatment and an experimental group

Blood collecting was performed from the measurement of body weight of a mouse, and a caudal vein before the experiment, and the blood sugar level was measured. The group division of the mouse was carried out so that weight and the blood sugar level might become almost equal for every group. An experimental group is a 1db/+m mouse control group, 2 db/db mouse control group, and 3 db/db mouse. It considered as the compound A (0.01%) administration group, and used each 7-10 groups at a time for the experiment.

b) A medication method and a period

Administration of compound A was performed by adding compound A by 0.01% of concentration as a powdered bait (F2, made in the Funabashi farm) (mixed feed administration). Compound A non-adding food was given to the control group.

c) An experiment period and blood collecting, dissection, RA Mr. island isolation

From 6 weeks old, the experiment period started administration, performed blood collecting and measurement of body weight during the morning eight days after, and dissected in the afternoon on the same day. A mouse makes an incision in the abdomen after decapitation. The vein needle of 27G-30G It uses and is HBSS () from a pancreatic duct to the pancreas. [Hank's] Balanced Salt Solution : P/N 24020-117 or KRB Product made from Gibco BRL (Krebs ringer buffer pH 7.4 : 129 mM NaCl, 4.8 mM KCl, and 1.2 mM MgSO4 --) 1.2mM KH2PO4, 2.5mM CaCl2, 5mM NaHCO3, 10mM HEPES pH 7.4, and 4 mg/ml CollagenaseType XI (P/N C7657 made from Sigma) dissolved in 0.2 % BSA About 2.5ml flowed in. Then, the pancreas were extracted in the 50ml falcon tube, and it incubated for 3 minutes and 30 seconds at 37 degrees C. Subsequently, cooled HBSS or 30ml of KRB(s) were added, and it put for 4 minutes after pipetting quietly in Hikami. About 25ml of supernatant liquid was removed, HBSS cooled again or 30ml of KRB(s) were added, and it put for 3 minutes after pipetting quietly in Hikami. About 29ml of supernatant liquid was removed (it leaves

about 6ml), and the remaining suspended solid was filtered to two Bacterial dish using the tea strainer. RA Mr. islands were gathered and collected under the stereoscopic microscope using the pipet man (P-10). After summarizing the extracted RA Mr. island in each one group, it dissolved by Trizole reagent (P/N 15596-018 made from Gibco BRL), and it was carried out to the RNA extract.

d) Measurement of a blood biochemistry value (a parameter and measuring method)

The parameter was taken as the blood sugar level, weight, and insulin concentration in blood. The blood sugar level was measured using the GURUKO loader GXT (product made from A&T). Moreover, insulin concentration in blood was measured by the radioimmunoassay method using RAT INSULIN RIA KIT (LINCO Research, product made from Inc.). A result is shown in Table 1 and drawing 1 (A-C).

[0149]

[Table 1]

		Plasma Glucose (mg/dl)		Body Weight (g)		Plasma Insulin (ng/ml)	
		Time (days)		Time (days)		Time (days)	
		0	8	0	8	0	8
Mean	db/+m	194.1	170.5	22.8	24.5	1.4	2.8
	db/db	374.5	523.7	29.9	34.8	20.8	13.3
	化合物 A (0.01%)	374.3	196.6	30.2	37.7	24.2	4.5
SE	db/+m	4.2	3.5	0.3	0.3	0.2	0.4
	db/db	20.5	22.5	0.3	0.4	1.8	1.1
	化合物 A (0.01%)	22.7	9.5	0.5	0.5	3.9	0.7

[0150]

e) Evaluation

Evaluation of drug effect followed each item of the blood sugar level, the insulin concentration in plasma, and weight.

[0151]

4. Result

As shown in drawing 1 (A-C), in the db/db mouse control group, the symptom characteristic of type 2 diabetes, such as a rise of the remarkable blood sugar level and a fall of plasma insulin concentration, was seen. On the other hand, it is a db/db mouse. By the compound A (0.01%) administration group, normalization of plasma insulin concentration and the blood sugar level was seen, and the improvement of the beta cell function by improvement of insulin resistance was suggested.

[0152]

[Example 2] Extract of all RNA from a RA Mr. island

The RA Mr. island collected in the example 1 added the chloroform of 0.2 capacity after dissolving in Trizole reagent (P/N 15596-018 made from Gibco BRL), and carried out fall mixing for 15 seconds. Subsequently, after putting for 10 minutes at the room temperature, at-long-intervals alignment separation was carried out at 12,000xg and 4 degrees C for 15 minutes. The upper layers were collected after centrifugal separation, and the RNase non-** isopropyl alcohol of 0.8 capacity was added, and it mixed with it. After carrying out at-long-intervals alignment separation of this at 12,000xg and 4 degrees C after standing for 10 minutes with a room temperature for 15 minutes,

supernatant liquid was removed and 80% ethanol of RNase non-** was added. Furthermore, all RNA was obtained by carrying out at-long-intervals alignment separation of this at 12,000xg and 4 degrees C for 10 minutes, removing supernatant liquid, and drying precipitate. All obtained RNA checked whenever [purification] by checking the wave of 28S and 18S using the Agilent 2100 biotechnology ANARAI riser (product made from Agilent technologies). All RNA saved at -80 degrees C till use.

[0153]

[Example 3] GeneChipTM analysis

1. Test Method

Chip analysis was performed by the following approaches according to the manifestation analysis engineering manual (Expression Analysis Technical Manual) of an AFI metrics company.

a) Composition of cDNA

According to the approach given [above-mentioned] in a manual, composition and purification of cDNA were performed by using as a start ingredient RNA each 5microg of all obtained in the above-mentioned example 2.

b) Composition of cRNA

According to the approach given [above-mentioned] in a manual, cRNA was produced by using as mold cDNA obtained by Above a. Obtained cRNA It fragmented and 10microg was added to the probe solution.

c) Production of a probe solution

The various control cRNA (GeneChipTM Eukaryotic Hybridization Control Kit) added to a probe solution was purchased from Pharmacia Corp., Amersham.

d) Hybridization

as the probe obtained by Above c, and the chip made to hybridize -- AFI metrics company make -- the mouse genome U74 set (Murine Genome U74 ver.2 Set:MG-U74Av2, MG-U74Bv2, MG-U74Cv2) was used. Washing actuation of hybridization and after that was performed according to the above-mentioned manual publication (hybridization conditions were made into 45 degrees C and 16 to 20 hours).

e) Analysis

Data analysis of the chip which performed hybridization actuation by Above d was performed by GeneChipTM Microarray Suite 4.0 (Affymetrix) according to the above-mentioned manual publication. In addition, "the Fold Change value (henceforth a Fold value)" showing the amount of relative manifestations of each gene showed gene expression level. They are a db/db mouse control group and a db/db mouse on the basis of the data of a db/+m mouse control group. Comparison examination of the data obtained by the compound A administration group was carried out.

[0154]

And in 74vMG-U2 chip, as compared with the db/+m mouse control group, when a manifestation medicated a db/db mouse with compound A administration in a high gene by the db/db mouse control group, the gene which gene expression normalizes was extracted. In addition, it was presupposed in the extract that the gene relation with diabetes mellitus is proved beforehand that it is is removed.

[0155]

2. Result

Eight genes as shown in Table 2 were newly specified as a gene which is a gene high-discovered with the diabetes-mellitus model mouse (db/db mouse control group) as a result, and the manifestation normalizes by insulin resistance improvement agent compound A administration.

[0156]

[Table 2]

特定された遺伝子の相対的発現量（Fold 値）

Gembank Accession No.	Annotation	配列番号		Fold 値	Fold 値
		塩基配列	アミノ酸配列	Ho/He*	化合物 A /He**
AK005296	AK005296	配列番号 1	配列番号 9	3.13	1.36
AK005484	Zsig37	配列番号 2	配列番号 10	3.43	1.09
NM_021434	hypothetical protein	配列番号 3	配列番号 11	3.37	1.33
AK006207	AK006207	配列番号 4	配列番号 12	3.94	1.15
BC010831	BC010831	配列番号 5	配列番号 13	3.01	1.13
AK013996	AK013996	配列番号 6	配列番号 14	2.53	0.97
AF272044	BR13	配列番号 7	配列番号 15	2.60	1.19
NM_007786	κcasein	配列番号 8	配列番号 16	15.43	1.36

* Ho (db/db マウス) /He (db/+m マウス)

**化合物 A (db/db マウス 化合物 A 投与群) /He (db/+m マウス)

[0157]

[Example 4] Gene expression analysis by TaqMan PCR

TaqMan PCR was used for eight gene ****** and the pan which were specified in the example 3, and the amount of manifestations was analyzed.

1. Test Method

a) Composition of cDNA

(SuperScript Preamplification System : Use P/N 18089-011 made from Gibco BRL for the following experiments)

By using as a start ingredient all RNA of 1microeach g obtained in the above-mentioned example 2, water was added to 10 x Reaction buffer 1microl and DNaseI (1 U/mu l : Gibco BRL P/N 18068-015) 1U, and it was referred to as whole-quantity 10microl, and incubated for 15 minutes at the room temperature. Next, it ice-cooled, after adding 25mM EDTA 1microl and incubating for 15 minutes at 65 degrees C. It ice-cooled, after incubating for 10 minutes at 70 degrees C Oligo(dT) (0.5microg/mul) 1microl In addition to this. Next, 10 x PCR buffer 2microl, 25mM MgCl2 2microl, 10mM dNTP mix 1microl, 0.1M DTT 2microl, and SuperScript II RT (200U/mul) 1microl were added, and it incubated at 70 more degrees C by 42 degrees C for 15 minutes for 50 minutes. RNase H 1microl In addition to this, it incubated for 20 minutes at 37 degrees C, and cDNA was produced. About the sample for standard, water was 29microl Added and this was diluted with dilution to six steps 5 times (concentration of an undiluted solution was set to 625). 229microl Water was added about the sample for measurement.

Furthermore, it is the beta-cells-of-pancreas origin because of a comparison. Culture system cell The sample was similarly adjusted using MIN6.

[0158]

b) Adjustment of reaction mixture

Upper primer (100microM) 0.1microl, Lower primer (100microM) 0.1microl, TaqMan Probe (6.5microM) 1.5microl, 2 x TaqMan Universal PCR Master Mix (PE ABI P/N 430447) 25microl, and water cDNA 5microl it produced by the above-mentioned a in the reaction mixture which mixed 21.3microl — it added. What was compounded at Invitrogen was used for the primer, and what was compounded with the FAM label by Amersham was used for the probe.

[0159]

It reaches each Primer. The array of Probe is shown below.

i) The primer for AK005296, and probe
Upper Primer: 5'-TACTGACCCGAGAAGCAGCA-3' (array number 17)
Lower Primer: 5'-CAGCTCTACATCAAATGCCCA-3' (array number 18)
Probe : (FAM label) 5'-CGGACCTTCTCGTCTCTGCACATTGA-3' (array number 33)

ii) Zsig37 The ** (AF192499) primer and probe
Upper Primer: 5'-TCCACCCCAGATCAACATCA-3' (array number 19)
Lower Primer: 5'-TTTTGCCGTACTTCCCCTG-3' (array number 20)
Probe : (FAM label) 5'-CATCCTGAAAGGCGAGAAAGGTGACC-3' (array number 34)

iii) hypothetical protein The ** (NM_021434) primer and probe
Upper Primer: 5'-GACATTGCCTCCCAAATTCA-3' (array number 21)
Lower Primer: 5'-CATCCGCACTATTGTCCAGC-3' (array number 22)
Probe : (FAM label) 5'-ATGCTGTACCTGCTTCTGAGCCTGTGTATG-3' (array number 35)

iv) The primer for AK006207, and probe
Upper Primer: 5'-CCTGTATTTCCAAGCTCTGCG-3' (array number 23)
Lower Primer: 5'-ATCCCAGAGCAAACACCACA-3' (array number 24)
Probe : (FAM label) 5'-ACATTTGTCCATGAAAGCCCTGCCTT-3' (array number 36)

v) The primer for BC010831, and probe
Upper Primer: 5'-CACAACCCACCCACATTGAT-3' (array number 25)
Lower Primer: 5'-TCCTTAGCAATGAGCATCCG-3' (array number 26)
Probe : (FAM label) 5'-ACCAGGAGTCTGCTCTGGCCAAACTT-3' (array number 37)

vi) The primer for AK013996, and probe
Upper Primer: 5'-ATCCTACTGGTGCTGAGTTCCC-3' (array number 27)
Lower Primer: 5'-GTGGTAATGCACACAATGCAGA-3' (array number 28)
Probe : (FAM label) 5'-TACCTGGCCTGGATCCTGTTCTTTGTGTT-3' (array number 38)

vii) BR13 The ** (AF272044) primer and probe
Upper Primer: 5'-AGGCGGATCAACAAACGTG-3' (array number 29)
Lower Primer: 5'-TGAGCGTCTCCACCACAAAT-3' (array number 30)
Probe : (FAM label) 5'-ACGCCATCCGCCACTTCGAGAATA-3' (array number 39)

viii) Kappa-casein (NM_007786) The ** primer and probe
Upper Primer: 5'-CTGCTGGAGTACCTTATGCCA-3' (array number 31)
Lower Primer: 5'-GGCGGTGTTATCCTGATTTTC-3' (array number 32)
Probe : (FAM label) 5'-CAAACCCATCCTTTCTTGCCATGCC-3' (array number 40)
[0160]
c) A reaction and measurement
After making the sample adjusted for the preceding clause b react at 95 degrees C by 50 degrees C for 10 minutes for 2 minutes, 1 minute was repeated 40 times at 60 degree C for 15 seconds by 95 degrees C, and it measured using the amount ABI PRISM 7700 of luminescence of reporter coloring matter for every cycle.
d) Analysis
The relative amount of manifestations of each gene was calculated using ABI PRISM 7700, and what amended them in the amount of beta-actin gene expression was used for analysis. A result is shown in Table 3 and drawing 2 - drawing 9 .
[0161]
[Table 3]

TaqMan PCR による各遺伝子の発現解析結果

	AK005296	Zsig37	Hypo pro.	AK006207	BC010831	AK013996	BR13	κ-casein
db/+m	0.0312	0.0012	0.0471	0.2012	0.0491	0.0121	0.0565	1.08028
db/db	0.1334	0.0470	0.1243	0.6894	0.2453	0.0432	0.1869	1.95300
化合物A	0.0510	0.0168	0.0677	0.3361	0.0987	0.0174	0.0968	0.79677
MIN6	0.0257	0.0024	0.0346	0.0735	0.0311	0.0000	0.0519	0.24740

[0162]
2. Result
Eight genes specified in the example 3 showed the notably high amount of manifestations by the diabetes-mellitus model mouse (db/db mouse) group as compared with the normal mouse (db/+m mouse) group and the beta cell origin culture system cell (MIN6) so that more clearly than Table 3 and drawing 2 -9. On the other hand, by the compound A administration group, it was checked that the amount of these gene expression is normalized more.
[0163]
From the above result, it was shown that the amount of gene expression shown according to the base sequence of the array numbers 1-8 can serve as diabetic symptoms and an index of the improvement, especially a beta cell malfunction improvement.
[0164]
[Example 5] The onset of the beta cell malfunction in a C57 BL/KsJ db/db mouse (drugs un-prescribing a medicine for the patient)
The blood biochemistry value of a type 2 diabetes model mouse was measured among 5 weeks old - 13 weeks old, and the onset of the beta cell malfunction accompanying the insulin resistance and this in the mouse concerned was observed.
[0165]
1. Animal
The male C57 BL/KsJ db/db mouse was used as a type 2 diabetes model mouse, and the male C57 BL/KsJ db/+m mouse (both made in Japanese Clare) was used as a normal mouse (contrast). After purchasing and acclimating a mouse by 5 weeks old, the experiment was presented with it by each week-old (5, 6, 7, 8, 10, 11, 12 or 13 weeks old). As for the mouse, during habituation / administration period considered drinking water and baiting (F2, Funabashi farm) as free intake. Breeding and the experiment during an administration period were conducted in the laboratory animal area managed by the laboratory animal management office.
[0166]
2. Test Method
a) An experiment period and blood collecting
The experiment performed measurement of body weight during the morning of each week-old, and blood collecting was performed after the 5-hour fast. Each group used 5-10 animals for measurement.
b) Measurement of a blood biochemistry value (a parameter and measuring method)
The parameter considered as the blood sugar level, weight, and insulin concentration in blood, and measured by the same approach as an example 1 (Table 4).
c) Evaluation
Evaluation of drug effect followed each item of the blood sugar level, the insulin concentration in plasma, and weight.
[0167]
[Table 4]

	血糖値		SE		血中インスリン値		SE		体重		SE	
週齢	db/db	db/+m	db/db	db/+m	db/db	db/+m	db/db	db/+m	db/db	db/+m	db/db	db/+m
5	143	160	25	23	5.4	0.8	1.4	0.2	28.2	22.9	0.7	1.3
6	250	158	69	17	10.7	0.7	3.9	0.1	34.8	24.1	1.6	1.2
7	282	140	93	28	11.6	0.9	4.9	0.2	34.5	25.3	1.1	1.3
8	533	138	123	33	10.4	0.7	6.7	0.1	39	25.2	1.8	1.7
10	554.4	109.6	144	22.7	3	0.8	1.6	0.3	48	28.2	4.2	1.1
11	608	137.6	65.1	11.9	3.6	0.7	1.8	0.2	46.1	30.6	1.6	1.9
12	665.6	134.4	63.8	18.5	3.5	1.1	0.9	0.5	50.5	27.7	0.7	1.1
13	762	132.8	68.1	20.1	5	0.5	1.4	0.2	43.5	29	2.5	2.2

[0168]
3. Result
In the type 2 diabetes model mouse (db/db mouse), the blood sugar level which was normal at 5 weeks old rose rapidly by 8 weeks old, and, also after that, increased quietly (drawing 10 A). In the db/db mouse, although what was about 10 ng(s)/ml in 6-8 weeks old fell to about 3 ng(s)/ml in 10 weeks old and the insulin concentration in plasma maintained the after that almost fixed value, it was a high price far as compared with the normal mouse (db/+m mouse) (drawing 10 B). From the above result, the db/db mouse showed insulin resistance in connection with aging, and causing a beta cell malfunction after that was checked.
[0169]
[Example 6] Cloning of a gene
1. Gene Which Carries Out Cloning
Zsig37 (array number 2) and kappa-casein (array number 8)
2. Approach
Cloning of Zsig37 gene and the kappa-casein gene was carried out to pcDNA3.1 Directional TOPO Expression Kit (Invitrogen, K4900-01) which is an expression vector in a mammals cell. The Zsig37 gene specific primer (Primer-1) and the kappa-casein gene specific primer (Primer-2) were produced, and the target gene was amplified by the PCR reaction using TOYOBO KOD-Plus (Toyobo KOD-201) by using as mold cDNA adjusted in the example 3. The primer produced what was designed so that an ORF overall length might be amplified.
[0170]
Zsig37 gene specific primer (Primer-1)
Upper primer: 5'-GAAGAGACGCCTCCCCGAGAGC-3' (array number 41)
Lower primer: 5'-TGCCAGGCAAGGAGGTCAGCAGTCC-3' (array number 42)
Kappa-casein gene specific primer (Primer-2)
Upper primer: 5'-CCAAATAAAGGTGCAATGATG-3' (array number 43)
Lower primer: 5'-CTTAGTGTTTTATGCTGCAGT-3' (array number 44)
[0171]
An PCR reaction is cDNA 2microl and Upper primer (20microM) 5. mul, Lower primer (20microM) 5microl, 10 x KOD Buffer 5microl, dNTP Mixture 5 (each 2.5mM) mul, MgSO4 2microl and TOYOBO KOD-Plus-Taq (1U) 1 mul and water the reaction mixture which mixed 25microl -- after 95 degrees C and pre-heat treatment for 1 minute, by 95 degrees C, for 30 seconds was repeated at 55 degrees C for 30 seconds, and this was repeated for for 1 minute 35 times as 1 cycle at 72 degrees C. an increase -- a by-product -- 1% agarose gel -- the increase after electrophoresis and of the bottom of UV irradiation -- a by-product -- it started and extracted using QIAquick Gel Extraction Kit (QIAGEN 28706 (250)). Extracted gene fragment 4microl, pCR-Blunt II-TOPO(Invitrogen) 1microl, and Salt Solution 1microl were mixed, and the ligation reaction was

performed by leaving it for 5 minutes at a room temperature. Transformation of the ligation reaction mixture was carried out to the Escherichia coli DH5alpha stock, and the clone was obtained by using ampicillin resistance as a marker ("Molecular Cloning and A Laboratory Manual" Maniatis, T., Fritsch, E.F., Sambrook, J.(1989) Cold Spring Harbor Laboratory Press). The check of the base sequence of the obtained clone used Sequencer (ABI PRISM 3700 DNA ANLYZER). The clone containing the target ORF was acquired. Next, the gene except a stop codon array was amplified by the PCR reaction by using this clone as mold using TOYOBO KOD-Plus (Toyobo KOD-201). As a primer which amplifies the part except a stop codon array, the Zsig37 gene specific primer (Primer-3) and the kappa-casein gene specific primer (Primer-4) were used.

[0172]

Zsig37 gene specific primer (Primer-3)

Upper primer: 5'-CACCATGGGCTCCTGTGCACAGGG-3' (array number 45)

Lower primer: 5'-GGGCTCAGAGGCTGGCTTGA-3' (array number 46)

Kappa-casein gene specific primer (Primer-4)

Upper primer: 5'-CACCATGATGAGGAATTTTATCGTAG-3' (array number 47)

Lower primer: 5'-TGCTGCAGTTGAGGACACTGGG-3' (array number 48)

[0173]

An PCR reaction cDNA 2microl, Forward primer (20microM) 5microl, Reverse primer (20microM) 5microl, 10 x KOD Buffer 5microl, dNTP Mixture (each 2.5mM) 5microl, MgSO4 2microl, TOYOBO KOD-Plus-Taq (1U) 1microl, and water the reaction mixture which mixed 25microl -- after 95 degrees C and pre-heat treatment for 1 minute, by 95 degrees C, for 30 seconds was repeated at 55 degrees C for 30 seconds, and this was repeated for for 1 minute 35 times as 1 cycle at 72 degrees C. an increase -- a by-product -- 1% agarose gel -- the increase after electrophoresis and of the bottom of UV irradiation -- a by-product -- it started and extracted using QIAquick Gel Extraction Kit (QIAGEN 28706 (250)). Extracted gene fragment 4microl, pcDNA3.1 D/V5-His-TOPO vector (Invitrogen, K4900-01) 1microl, and Salt Solution 1 mul was mixed and the ligation reaction was performed by leaving it for 5 minutes at a room temperature. Transformation of the ligation reaction mixture was carried out to the Escherichia coli DH5alpha stock, and the clone was obtained by using ampicillin resistance as a marker ("Molecular Cloning and A Laboratory Manual" Maniatis, T., Fritsch, E.F., Sambrook, J.(1989) Cold Spring Harbor Laboratory Press). The base sequence of the obtained clone was checked using Sequencer (ABI PRISM 3700 DNA ANLYZER). In this way, the clone containing the target ORF was acquired.

[0174]

[Example 7] Secretion check

1. Used Gene

Zsig37 (array number 2) and kappa-casein (array number 8)

2. Approach

2.1 Installation of Gene, and Recovery of Culture Supernatant

a) The 1st day

On 6cm petri dish, COS-1 cell suspended in FBS/DMEM 10% was scattered so that it might become subcontractor FURENTO on the next day, and it was cultivated under CO2 existence 5% 37 degrees C on it at inside on the morning of the 1st.

b) The 2nd day

DMEM-FBS after washing twice COS-1 cell prepared on the previous day by DMEM-FBS (-) (-) 2 ml was added and it prepared as a cell for lipofetion.

[0175]

He is an end free-lancer beforehand. Plasmid MIDI The Zsig37 manifestation plasmid DNA water solution of 4.0microg refined using the kit (QIAGEN) was set to 234microl by DMEM-FBS (-), 16microl addition of PLUS reagent was done further, and it incubated at the room temperature after mixing for 15 minutes. Next, it is DMEM/FBS (-) beforehand. LipofetAMIN mixed liquor which added and produced LipofectAMIN 24microl to 226microl After adding 250microl and mixing with it, it considered as the DNA solution for lipofection by incubating at a room temperature for 15 minutes.

The DNA solution for lipofection was slowly dropped at the cell for Lipofection, and 37 degrees C was cultivated under CO2 existence 5% for 4 hours. Then, it exchanged for FBS/DMEM 0% and 37 degrees C was cultivated for three days under CO2 existence 5%.
c) The 5th day
Culture supernatants were collected.
[0176]
The manifestation check of 2.2 proteins
a) To 1ml of culture supernatants, 4mg/ml DOC in TCA 100microl was added, and fall mixing was carried out for 15 seconds. Subsequently, after putting for 5 minutes at the room temperature, at-long-intervals alignment separation was carried out by 15,000xg for 5 minutes. Cold acetone 500microl was added to precipitate after centrifugal separation, and fall mixing was carried out for 15 seconds. Subsequently, at-long-intervals alignment separation was carried out by 15,000xg for 5 minutes. Cold acetone 500microl was again added to precipitate after centrifugal separation, and fall mixing was carried out for 15 seconds. Subsequently, at-long-intervals alignment separation was carried out by 15,000xg for 5 minutes. After centrifugal separation, supernatant liquid was removed, precipitate was dried, 1x SDS sample beffer 20microl was added, and it boiled for 5 minutes at 100 degrees C, and considered as the sample for electrophoresis.
b) After whole-quantity applying, the electrophoresis buffer (Tris 15.15g, glycine 72.05g, distilled water 5L, SDS 5g) was used, and electrophoresis of the sample for electrophoresis was carried out to 5-20% gradient gel for 40 - 50 minutes by 40mA.
c) Next, blotting of the gel which carried out electrophoresis by 40V and 200mA using Brocin GUBAFFA (Tris 15.14g, glycine 72.07g, distilled water 3L, methanol 1L) was carried out to nitrocellulose membrane for 2 hours. membrane was dipped in the TBS-T solution after blotting termination.
d) membrane was moved to the skim milk/TBS-T solution 5%, and it shook on the shaker at the room temperature for 1 hour.
e) membrene -- a TBS-T solution -- 5 minutes x -- it washed by carrying out shaking shake 3 times.
f) membrane was moved to the primary antibody solution (anti-V5 antibody 2microl, BSA 0.1g, TBS-T 10ml), and it shook on the shaker at the room temperature for 1 hour. next, membrene -- a TBS-T solution -- 5 minutes x -- it washed by carrying out shaking shake 3 times.
g) membrane was moved to the second antibody solution (anti-mouse antibody 10microl, BSA 0.1g, TBS-T 10ml), and it shook on the shaker at the room temperature for 1 hour. next, membrene -- a TBS-T solution -- 5 minutes x -- it washed by carrying out shaking shake 3 times.
h) It is an ECL reagent on membrane. 2ml was carried and it was made to react for 1 minute at a room temperature.
i) membrane which removed the ECL reagent evaluated the signal by developing negatives with an auto-processor after exposing to a package and an X-ray film with Saran Wrap.
[0177]
3. Result
It was checked in the culture supernatant of the cell which carried out transfection of Zsig37 and kappa-casein that each protein is secreted (drawing 11).
[0178]
[Example 1 of reference] Manufacture of 5-[4-(6-methoxy-1-methylbenzimidazol-2-ylmethoxy) benzyl] thiazolidine -2 and 4-dione hydrochloride (compound A)
5-[4-(6-methoxy 1-methylbenzimidazol-2-ylmethoxy) benzyl] thiazolidine [which was compounded by the approach of a publication to JP,9-295970,A (a U.S. Pat. No. 5886014 specification, the Europe patent application public presentation No. 745600 specification)] -2 and 4-dione 10.6g and 4 convention hydrochloric acid -1, and 4-JIOSAKIN 100ml mixture were agitated at the room temperature for 1 hour. The results object which added and ****(ed) ethyl acetate was separated after condensing a reaction mixture, ethyl acetate washed, and 11.0g of compound A which has the 275 to 277 degree C melting point was obtained.
[0179]

1H-nuclear-magnetic-resonance spectrum: 1H-nuclear-magnetic-resonance spectrum (400MHz):delta (ppm) measured to the internal standard using TMS (tetramethylsilane) is as follows among delta(ppm):pile dimethyl sulfoxide.

[0180]

3.11 (1H, dd, J= 14Hz, and 9Hz) 3.34 (1H, dd, J= 14Hz, and 4Hz), 3.89 (3H, s), 3.98 (3H, s), 4.91 (1H, dd, J= 9Hz, and 4Hz), 5.64 (2H, s), 7.14 (2H, d, J= 9Hz), 7.15 (1H, d, J= 9Hz), 7.25 (2H, d, J= 9Hz), 7.50 (1H, s), 7.70 (1H, d, and 9H), 12.04 (it disappears by 1H, s, and D2O addition).

[Availability on industry]

[0181]

The amount of gene expression concerning this invention shown according to any one base sequence of the array numbers 1-8 can serve as a new index for evaluating the beta cell malfunction improvement in diabetes mellitus. Therefore, simple screening of beta cell malfunction improvement agents, such as an insulin resistance improvement agent and an insulin secretion accelerator, can be performed by making the amount of manifestations of this gene or its product into an index.

[Array table free text]

[0182]

Explanation of the array number 17 - a 32-artificial array: Primer

Explanation of the array number 33 - a 40-artificial array: Probe

Explanation of the array number 41 - a 48-artificial array: Primer

[Brief Description of the Drawings]

[0183]

[Drawing 1] Drawing 1 is a graph which shows the blood biochemistry value in each group, and the measurement result of weight (A: the blood sugar level, insulin concentration in B:plasma, C:weight).

[Drawing 2] Drawing 2 is a graph which shows the manifestation analysis result of AK005296(array number 1) mRNA by TaqMan PCR.

[Drawing 3] Drawing 3 is a graph which shows the manifestation analysis result of Zsig37(array number 2) mRNA by TaqMan PCR.

[Drawing 4] Drawing 4 is a graph which shows the manifestation analysis result of hypothetical protein(array number 3) mRNA by TaqMan PCR.

[Drawing 5] Drawing 5 is a graph which shows the manifestation analysis result of AK006207(array number 4) mRNA by TaqMan PCR.

[Drawing 6] Drawing 6 is a graph which shows the manifestation analysis result of BC010831(array number 5) mRNA by TaqMan PCR.

[Drawing 7] Drawing 7 is a graph which shows the manifestation analysis result of AK013996(array number 6) mRNA by TaqMan PCR.

[Drawing 8] Drawing 8 is a graph which shows the manifestation analysis result of BR13(array number 7) mRNA by TaqMan PCR.

[Drawing 9] Drawing 9 is a graph which shows the manifestation analysis result of kappa-casein (array number 8) mRNA by TaqMan PCR.

[Drawing 10] Drawing 10 is a graph which shows the blood biochemistry value in a type 2 diabetes model mouse and a normal mouse, and the measurement result of weight (A: the blood sugar level, insulin concentration in B:plasma, C:weight).

[Drawing 11] Drawing 11 is a photograph in which the secretion check result (Western blotting) of Zsig37 and kappa-casein protein is shown.

[Translation done.]

DESCRIPTION OF DRAWINGS

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[Translation done.]